METHOD FOR EXAMINING REACTIVITY AND METHOD FOR DETECTING A COMPLEX

This application is a division of U.S. Patent Application No. 09/942,662, filed August 31, 2001, now abandoned, which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention is directed to examining multiple specimens at a time for multiple items, and provides a method in which matrix substrates with biological samples having different properties and origins bound thereto are prepared, and on each matrix region, oligonucleotides having different sequences, proteins or drugs are spotted in an array, whereby multiple specimens are examined at a time for multiple items.

The present invention also relates to a method in which, by using an oligonucleotide having a known base sequence as a detection probe to detect whether a complex is formed by intermolecular bond with this oligonucleotide, detection is made as to whether or not components having a capability of bonding to the above-described detection probe are contained, and to a detection substrate having the oligonucleotide as a detection probe fixed on its surface, which is used exclusively for this detecting method.

Related Background Art

In identification of partial sequences included in the base sequence of a nucleic acid molecule, detection of a target nucleic acid contained in a sample originated from an organism or identification of genus or species for various bacteria based on the characteristics of the gene DNA of the

bacteria, a procedure may be used in which two or more probe DNAs having known base sequences are used to detect whether or not the nucleic acid molecule is a nucleic acid molecule specifically binding to each probe DNA, namely making hybridization with each probe DNA. As an effective approach to performing speedily and accurately examination of the two or more probe DNAs by the hybridization method, a procedure is proposed in which a probe array of two or more probe DNAs arranged regularly on a solid phase is used to detect at a time whether or not the nucleic acid molecule is a nucleic acid molecule specifically binding to each probe DNA.

Among common methods for producing such probe arrays, as described in European Patent No. 373203 (EP 0373203 Bl) for example, methods are known in which predetermined nucleic acid probes are synthesized in an array form on a solid phase, and methods in which a plurality of nucleic acid probes synthesized in advance is supplied in an array form on the solid phase.

Prior technical documents disclosing the former methods include, for example, U.S. Patent No. 5,405,783. Also, as one example of the latter methods, a method in which cDNAs are arranged in an array form on a solid phase using micropipetting is disclosed in, for example, U.S. Patent No. 5,601,980 and "Science", Vol. 270, pp. 467, (1995).

The probe array that is prepared using these methods may be an array in which nucleic acid probes are arranged on a solid phase at a high density of 10000 or more probes per square inch. A hybridization reaction with multiple probes is carried out at a time by dipping this high-density probe array into a specimen solution, and in so doing, the base sequence of genes is analyzed based on the base sequence of nucleic acids that hybridize. This method has an advantage in that

probes are arranged at a high density on a substrate with a small area, thereby making it possible to conduct a multiple-item examination at the same time with a small amount of samples to reduce the burden associated with sampling from the subject.

As a method of preparing the high-density probe array for the above-described application on the substrate by the DNA synthesis process, a method in which a photolithography technology is applied is disclosed in the aforesaid U.S. Patent No. 5,405,783, but highly advanced equipment is required for implementing this method, and the method is not easy enough for anyone to use.

Also, in the case where the number of specimens is large but the number of required examination items is not so large, the integration degree of DNA probes on the probe array corresponding to the number of examination items does not need to be very high. Rather, there may be cases where it is necessary to prepare a large number of probe arrays with a small number of desired DNA probes fixed, using a simpler method.

Actually, in the field of clinical examination, there are not necessarily many cases where examinations for more than 10000 items are required. For example, in the case of group health examination and the like, there may be cases where it is more important to examine a large number of specimens with a limited number of items. For examining a large number of specimens in this way, a system is required such that presence of diseases can be speedily examined through comparison with standard samples with respect to each specimen.

In addition, the amount of a DNA specimen is generally small as compared to that of an oligonucleotide capable of being synthesized and used in the probe. For using it in a

normal form in which the probe array substrate is dipped into the specimen solution for a hybridization reaction, the amount of specimen DNA allowing the substrate to be dipped sufficiently is required. Therefore, the size of the DNA probe array substrate is limited depending on the amount of specimen DNA, and thus the array needs to be highly dense. Alternatively, as a result of diluting the specimen solution to ensure its volume for the size of the probe array substrate, the concentration of DNA in the specimen solution is reduced, and a procedure is adopted for prolonging the reaction time to compensate for the reduced concentration.

Also, since the amount of sampled specimens is limited inherently because the specimen is an extract from tissues, and because it is subjected to pre-processing for making a specimen solution for use in the hybridization reaction, specifically, extraction of nucleic acid, single-strand formation thereof, and process for labeling, the amount of finally obtained samples is very small. In order to compensate for that, the sample is subjected to processing for amplification of the amount of DNA, such as amplification processing by PCR reaction, before it is used for examination and studies. However, there exists a disadvantage in that because separately prepared primers are required for carrying out a PCR reaction, such processing can be applied only to specific genes of which primer sequence is known. In addition, there exist sequences that can easily be amplified and sequences that can hardly be amplified in the process of PCR reaction, and thus, the efficiency of reaction (rate of amplification) is not uniform. For example, in the case where the content of a specific mRNA in the total amount of extracted mRNA is examined to determine diseases or like based on the content, standard samples providing criteria should be

always prepared to make correction on the above-described amplification rate.

Although the amount of the specimen solution required for a hybridization reaction decreases as the size of the substrate is reduced, there is a limitation on downsizing of the substrate in association with handling. Specifically, it is possible in principle to enhance array density and reduce the number of probes to be placed on the array to downsize the substrate, but if an extremely small substrate is used, a dedicated handling apparatus is required in the process of processing such as hybridization reaction and detection thereafter, which cannot be practical.

Also, for examining cDNA for mRNA that is transcribed with reflection of the process of development of a certain organism, cDNA for mRNA that is transcribed with reflection of each phase in the process of culturing a certain cell, cDNA for mRNA that is transcribed by interaction with drugs, and so on, a DNA array with multiple types of test samples arranged is used. Examples of arraying this test sample are described, for example, in the above-described "Science", Vol. 270, pp 467, (1995). In this case, test samples arrayed on the substrate are dipped using as a probe solution the labeled DNA of known sequence that is derived from genes having a specific function, whereby a hybridization reaction is carried out.

If a plurality of items is to be examined at a time using this methodology, DNA probes labeled with different types of fluorescent reagents (fluorochromes) should be prepared depending on the number of items. When a detection is made, those different types of fluorescent reagents (fluorochromes) must be distinguished from one another, and therefore, their wavelengths and the like should be different as a matter of course. Of course, detection filters corresponding to

respective fluorescent reagents (fluorochromes) are also needed for a detector.

This need for a simultaneous examination of multiple items for multiple specimens is not characteristic exclusively of a hybridization reaction among genes (DNA).

For example, it is also important to examine multiple items with a small amount of samples as to the interaction between genes and other substances, such as interaction between genes and proteins (DNA binding proteins) and screening of chemicals that are bound to genes. Detection of former DNA binding proteins is used to elucidate the control mechanism of gene expression by proteins, such as transcription accelerators. However, in the present situation, methods in which DNA fragments are bound to proteins, and thereafter complexes are analyzed by gel electrophoresis, are adopted. In this method, the number of specimens that can be analyzed at a time is limited due to the usage of gel electrophoresis, and considerable time is required for analysis.

For the field of drug development, there may be cases where an examination of interaction between genes and administered drugs constitutes an important item in the progress of research, but it takes relatively a large amount of time and effort to obtain chemically synthesized products for use in drugs to be researched, and it can be considered that a reduction in the amount of drugs to be used in screening results is a significant improvement in efficiency of their research.

As introduced above, there are cases where when a complex is formed using an interaction between two substances, such as hybridization between DNAs, formation of a complex of DNA and a protein, and interaction of a drug compound with gene DNA,

or the presence or absence of the interaction causing a complex to be formed, is examined, the amount of samples of one of those two substances is limited, and the limited amount of samples should be used to conduct a series of desired examinations across multiple types as to the presence or absence of formed complexes. That is, development of an examination method in which consumption of samples required for individual examinations can be reduced to carry out the examination across multiple types more efficiently within a limited amount of samples is desired.

SUMMARY OF THE INVENTION

An object of the first invention is to provide a method of examining multiple specimens at a time for multiple items, for example a method in which matrix substrates with biological samples having different properties and origins bound thereto are prepared, and on each matrix region, oligonucleotides or proteins having different sequences and drugs are spotted in an array form, whereby multiple specimens are examined at a time for multiple items.

Another object of the invention is to provide a method in which multiple specimens can also be examined at a time for multiple items in a similar way for interaction between chemicals, especially drugs, and cDNA, binding of proteins to cDNA and the like.

An object of the second invention is to provide a new method in which an oligonucleotide with a known base sequence and which can be obtained relatively easily is used as a detection probe. When, for a limited amount of sampled specimens, the presence or absence of a bonding capability to the above-described oligonucleotide as a detection probe or the degree of the bonding capability is examined by the

presence or absence of complexes formed between those two substances, or efficiency thereof is evaluated, consumption of specimens required for evaluation for each type of oligonucleotide as a detection probe can be reduced. In addition, the invention also has an object to provide a detection substrate with the above-described oligonucleotide being fixed as a detection probe in a predetermined region of its surface, which is used exclusively for the method, and to provide a method of preparing the detection substrate.

The examination method of the first invention capable of achieving the above-described objects is a method in which a reactivity between a first sample and a plurality of second samples having different properties from one another is examined at a time,

characterized in that in a defined region on a substrate with the first sample bound on the entire surface in advance, the second samples are placed independently of one another as spots having a smaller size than the above-described defined region, and then the reactivity between the above-described first sample and each of the second samples is tested.

The matrix of biological samples related to the invention that is usefully used for the above examination method is characterized in that two or more types of biological samples of different origins exist in respective matrix regions separated on the substrate.

According to the invention, a substrate with biological samples having different properties and origins (e.g. nucleic acids and proteins) bound in a matrix form in advance can be provided.

There is also provided a method in which DNA probes like oligonucleotides, cDNAs, proteins or chemicals are spotted in an array form on the above-described substrate with biological

samples having different properties and origins placed in a matrix form to carry out the reaction, and the presence or absence of another sample bound to a certain biological sample, the degree of the bonding, and the presence or absence of interaction is quickly examined for multiple items at the same time.

In this method, the area occupied by one specimen is very small, because two or more types of specimens are placed on one substrate. Therefore, there is an advantage in that the amount of required cDNA may be very small as compared to the case where the hybridization reaction is carried out using a conventional DNA array with an enormously large number of DNA probes bound in an array form in advance. Also, there is neither a limitation on the size of the DNA array substrate nor an inconvenience in handling.

Also, by providing a method in which examination can be carried out even with a small amount of samples, the method opens the door to areas in which examination could not be carried out, because conventionally, a sufficient amount of samples cannot be obtained, for example, a new examination area in which mRNA obtained from tissues is directly examined.

In addition, according to the invention, chemicals, proteins and nucleic acids can be examined at the same time under the same reaction condition on the same substrate.

A method of detecting object components in test samples according to the second aspect of the invention is a method in which using as a detection probe oligonucleotide with a known base sequence, complexes formed between the above-described oligonucleotide and the object components are detected to examine whether or not the object components capable of binding to the above-described oligonucleotide are contained

in the liquid test samples, or evaluate the degree of binding capability thereof,

characterized in that there is at least one type of the above-described oligonucleotide used as a detection probe with a known base sequence,

there are at least two types of test samples to be examined, and

a detection substrate with the above-described one or more types of oligonucleotide for detection probes bound to predetermined sections respectively on a predetermined solid substrate is used.

The above-described method comprises steps of:

spotting a plurality of predetermined amounts of sample solution for each spot so that a predetermined array shape is formed in the spotted position, for each of the above-described two or more types of test samples, in each section with the oligonucleotide to detect probes bound in advance;

detecting the presence or absence of complexes formed between the above-described oligonucleotide and the object component, for the above-described plurality of spots for each test sample, respectively; and

determining whether or not the object component capable of binding to the above-described oligonucleotide is contained, or the degree of the capability of binding, based on the result of the above-described detection.

Also, the present invention provides a detection substrate that is exclusively used when the above-described method of the invention is carried out. That is, the detection substrate of the present invention is a detection substrate with two or more oligonucleotides having known base sequences different from one another fixed on a solid substrate, characterized in that:

the above-described plurality of oligonucleotides are bound and fixed in predetermined sections, respectively, so that one type of oligonucleotide exists in each section, and

a plurality of the above-described sections in which oligonucleotides are fixed is placed in a matrix form on the surface of the above-described solid substrate.

The method of preparing the detection substrate of the present invention is a method suitable for preparation of the above-described detection substrate of the invention, and specifically, is a method of preparing a detection substrate with two or more oligonucleotides having known base sequences different from one another fixed on a solid substrate, characterized in that:

for the above-described solid substrate, a substrate with a plurality of sections separated in a matrix form in advance formed on the surface thereof is used,

the above-described plurality of oligonucleotides is supplied into predetermined sections in predetermined amounts using printing by an ink jet process, respectively, so that one oligonucleotide is present in each section, and

the supplied oligonucleotides are fixed in the predetermined sections.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows one example of an arrangement aspect of defined regions on a substrate in the present invention;

FIGS. 2A and 2B show one example of matrices in the present invention, wherein FIG. 2A is a plan view, and FIG. 2B is a 2B-2B sectional view thereof;

FIG. 3 is a schematic explanatory view of a specimen solution discharging method by a bubble jet process that is an embodiment of the present invention;

FIG. 4 is a sectional view of a bubble jet head 105 taken in the 4-4 line in FIG. 3;

FIG. 5 shows a layout of 64 discharged DNA probes on each black matrix;

FIG. 6 shows one example of detection substrates of the present invention, illustrating schematically a situation in which sections in which oligonucleotides being detection probes are fixed are arranged in a matrix form, and a plurality of cDNAs are spotted in a two-dimensional array form onto each section as detection samples;

FIG. 7 illustrates schematically arrangements of respective probes in the detection substrate with 64 DNA probes bound to sections arranged in the form of a 8 x 8 matrix, respectively;

FIG. 8 shows schematically a pattern of a spot 64 x 64 array in which 64 test samples are spotted in the form of a two-dimensional 8 x 8 array on each section, for the detection substrate on which sections with probes fixed therein are arranged in the form of the 8 x 8 matrix;

FIG. 9 shows schematically a result of spotting 64 test samples in the form of the two-dimensional 8 x 8 array on each section for 64 probes fixed in sections arranged in the form of the 8 x 8 matrix to carry out the hybridization reaction; and

FIG. 10 shows an example of the structure of sections delimited by hydrophobic frame-structured walls provided on the detection substrate of the present invention, and arranged in the form of the 8 x 8 matrix.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

One embodiment of the present invention will be described below referring to FIG. 1. FIG. 1 shows a substrate surface

with 64 defined regions formed thereon, wherein each region (matrix) measures 1 mm by 1 mm, and a space x between regions can be selected freely. For methods of preparing biological sample binding matrix substrates, for example, a method can be used in which the solution of a first sample (e.g. biological sample) is printed on the entire surface of defined regions on the substrate as a "solid print pattern" by coating and ink jet processes, or is supplied by methods, such as chemical synthesis on the substrate, and is bound in a matrix form on the substrate through adsorption to the substrate or chemical reaction between functional groups existing in the biological sample and functional groups existing on the substrate. Furthermore, the situation in which the first sample is bound on the entire surface of defined regions means a situation in which the first sample is bound across the entire surface, such that when a second sample and samples thereafter are supplied in these defined regions, these reactions occur without being limited to the positions in the above-described regions in which the samples are supplied. For example, the first sample may be fixed in layered form on the entire surface, or the masses of molecules constituting the first sample may be dispersed on the entire surface in high density with micro-spaces being kept among them.

The defined regions on this substrate may previously be provided on the substrate as a well constituted by sections separated in a pattern formed by walls of hydrophobic compounds.

Also, when using a substrate with a nucleic acid (cDNA) being a biological sample fixed thereon as the first sample, two or more probe DNAs possibly included in cDNA are contacted with cDNA on the substrate as the second sample and samples thereafter. Products of the reaction with the above-described

probes are detected on the above-described solid phase to detect the presence or absence of probe DNA sequences in the above-described cDNA, two or more probes are supplied in an array form as mutually independent spots in each matrix with various kinds of cDNA bound in the defined regions, thereby making it possible to perform simultaneous detection with two or more probes.

Also, on the nucleic acid (cDNA) matrix, two or more types of chemicals or proteins that are possibly bound to cDNA are contacted with the probe DNA on the substrate as mutually independent spots, thereby making it possible to perform a multiple-item examination composed of these reactions at the same time. Multiple-item screening of DNA binding proteins and DNA binding chemicals can be performed at the same time by detecting the presence of binding of chemicals or proteins to probes on the solid phase.

The present invention is characterized by supplying probe DNA, proteins and chemicals in a form of droplets of small amounts on the matrix on which biological samples, such as cDNA are applied, wherein different types of samples are arranged in an array form, thereby making it possible to perform simultaneous multiple-item processing.

Combinations of the first sample fixed in advance on the substrate and the second sample and sample thereafter that are reacted with the first sample may include the following combinations.

Specific examples of the matrix or the like formed of defined regions on the substrate for use in the present invention will be described below.

Shapes of Matrices with Biological Samples Bound Thereto

The shapes of matrix patterns are not particularly limited, but linear, square and rectangular shapes are

preferred in that they can be treated irrespective of how specimens are supplied. Of course, shapes such as circles and ellipses will cause no problems.

Materials that are fixed to the substrate as a first sample may include unknown base sequences derived from organisms, cDNA libraries, mRNA libraries, sets of two or more DNA and RNA, known DNA and RNA synthesized or derived from organisms or sets thereof, chips of cloned oncogenes, protein fractions including at least one type of protein derived from organisms, proteins of single type, mixtures of known proteins of different types, and chemicals.

Density of Matrices with Bound Biological Samples

The density of matrices is not particularly limited, but for a preferred form, the density of 400 per centimeter square is preferable. For this preferred density of $400/cm^2$, the size of one matrix is a 500 μm square in the case of a square shape. If samples to be arranged as spots on the array are 100 μm in diameter, 25 spots are arranged in total, 5 spots high by 5 spots wide. Also, if the diameter of sample solution is 20 μm, the number of spots that can be arranged in a row is 25, and 625 spots can be arranged in total.

Preparation of a Substrate with Biological Samples Bound Thereto

Samples originated from organisms (biological samples) include nucleic acids and proteins. Nucleic acids include, for example, mRNA and cDNA, and methods for binding them on the substrate include a method in which a nucleic acid extracted and purified in advance is applied to the substrate to fix the nucleic acid by adsorption and an electrostatic bond, and a method in which the nucleic acid is fixed by providing a covalent bond through a chemical reaction with

functional groups on the substrate using amino groups of the nucleic acid.

The method using negative electric charges of DNA is a method in which the nucleic acid is electrostatically bound to a solid carrier subjected to a surface treatment with positive polymeric ions, such as polylysine, polyethyleneimine and polyalkylamine, and then blocking of excessive positive ions is carried out, which is generally used.

Types of Functional Groups of Solid Phases and Nucleic Acids

Combinations of functional groups that are used for fixation include, for example, a combination of epoxy groups (on solid phase) and amino groups (amino groups in nucleic acid probe terminals or base groups). Methods for introducing epoxy groups to the solid surface include, for example, a method in which polyglycidyl methacrylate having epoxy groups is applied to the solid surface composed of resin, and a method in which a silane coupling agent having epoxy groups is applied to the solid surface made of glass and is reacted with glass.

Binding of Proteins to the Solid Phase

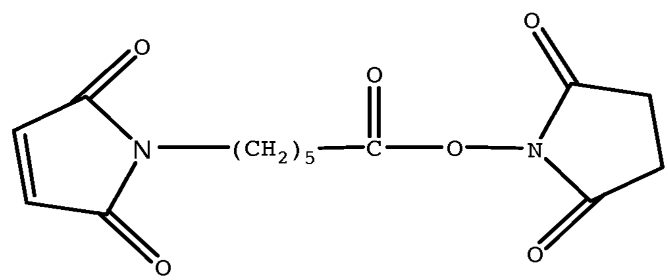
Methods of binding proteins to the substrate include methods using adsorption, as in the case of nucleic acid, and methods using electrostatic binding. Furthermore, methods of forming a covalent bond include those using SH groups of cysteine residues in addition to the above-described methods using amino groups.

Methods of Fixing Proteins Using Thiol Groups

Methods using cysteine residues for fixing proteins include, for example, methods using combinations of maleimide groups and thiol groups (-SH). That is, the treatment is performed so that the solid surface has maleimide groups,

whereby thiol groups of cysteine residues supplied to the solid surface can be reacted with maleimide groups of the solid surface to fix proteins.

Various kinds of methods may be used to introduce maleimide groups to the solid surface. This can be achieved by, for example, reacting an aminosilane coupling agent with a glass substrate, and then reacting its amino groups with a reagent containing N-(6-maleimidocaproyloxy) succinimide) expressed by the following structural formula (EMCS reagent: manufactured by Dojin Co., Ltd.)



.

As another example, a reagent containing succinimidyl 4-(maleimidophenyl) butyrate can be used to react with, preferably amino groups.

DNA Matrix Structures Composed of Hydrophobic Matrices

As an additional form of fixing biological samples, a method can be used in which a well composed of, for example, hydrophilic and hydrophobic matrices is formed on the solid surface, a structure to prevent coupling among spots is provided in advance, and the DNA prove is supplied in the well to carry out a coupling reaction.

Materials of Matrices/Wells

When a prove solution is put on the separated matrix to carry out the coupling reaction, it is preferable that portions constituting the well are hydrophilic, and portions corresponding to the wall surface of the well and the partition between the well and a neighboring well are composed

of materials whose surfaces are less compatible with the prove solution. Due to such a treatment, the probe solution can be smoothly supplied to a desired well even if some positional deviation occurs when the prove solution is supplied to the well.

One example of a matrix in this embodiment is shown in FIGS. 2A and 2B. FIG. 2A is a plan view, and FIG. 2B is a 2B-2B sectional view thereof. This matrix has a structure in which a matrix pattern 125 having a frame structure with formed recesses 127 (wells) placed in the form of a solid phase 103 is provided. The wells 127 separated from one another by the matrix 125 (height) are provided as through-holes (cut-off portions) in the matrix pattern. The side of the matrix pattern is constituted by height, and the bottom 129 has the exposed surface of the solid phase 103. The portion of the exposed surface of the solid phase 103 forms a surface that can be coupled to the probe, and the probe is fixed in a predetermined recess.

Materials forming the matrix pattern include, for example, metals (chrome, aluminum, gold, etc.) and resins. They include resins, such as acryl, polycarbonate, polystyrene, polyimide, acrylate monomers and urethane acrylate, and photosensitive resins, such as photoresists having black dies and black pigments contained therein. For specific examples of photosensitive resins, UV resists, DEEP-UV resists, ultraviolet cured resins and the like can be used. UV resists may include negative resists, such as cyclized polyisoprene-aromatic bisazide resists, phenol resin-aromatic azide compound resists, and positive resists, such as novolac resin-diazonaphthoquinone resists.

DEEP-UV resists may include, for example, radiation dispersion type polymer resists, such as polymethyl

methacrylate, polymethylene sulfone, polyhexafluorobutyl methacrylate, polymethyl isopropenyl ketone and bromo poly 1-trimethylcylilpropine, and dissolution inhibiting resists, such as cholate o-nitrobenzyl ester as positive type resists, and may include polyvinylphenol-3-3'-diazidediphenylsulfone, and polymethacrylate glycidyl as negative type resists.

Ultraviolet cured resins may include polyester acrylate, epoxy acrylate and urethane diacrylate containing approximately 2 to 10% by weight of one or more types of photopolymerization initiators, which are selected from benzophenone and substituted derivatives thereof, oxime compounds, such as benzyl, and so on.

For curbing reflection by the material forming the matrix during detection, light-blocking materials can be effectively used to form the matrix pattern. For this purpose, it is effective to add black pigments in the above-described resin. Examples of black pigments that can be used are carbon black and black organic pigments.

Here, if the matrix 125 is composed of resin, the surface of the matrix 125 is hydrophobic. This structure is preferred when an aqueous solution is used as a solution containing probes to be supplied to the well. That is, even if the prove solution is supplied to the well, the prove solution is supplied to a desired well quite smoothly. Also, if different probes are supplied among adjacent wells at the same time, intermingling (cross-contamination) of different probe solutions supplied among these wells can be prevented.

The thickness of the matrix (height from the solid surface) is determined in light of the matrix pattern forming process and the volume of the well, but it is preferably in the range of 1 to 20 μm. Particularly, it can be considered as a thickness range that effectively prevents cross-

contamination when the probe solution is supplied to each well through an ink jet process.

Types of Samples to be Spotted

Samples to be spotted as droplets onto the above-described matrices of biological samples include probe nucleic acids, proteins and chemicals, such as drugs.

For probe nucleic acids, in addition to deoxyribonucleic acid, any type of nucleic acid, such as ribonucleic acid and peptide nucleic acid, may be used as long as it has nucleic acid bases. The length of the oligonucleotide probe is not particularly limited, but it is preferably in the range of 10 mer to 50 mer for carrying out an accurate hybridization reaction with cDNA.

For proteins, their own fluorescence can be used to detect DNA bonding proteins.

Some chemicals can also be detected with their own fluorescence.

Method of Preparing Sample Arrays

Methods of spotting sample solution on defined positions in the size of several tens to several hundreds of microns include a pin system, an ink jet system and a capillary system.

The pin system refers to a method in which the sample is attached to the pin tip, for example, in such a manner that the pin tip is contacted with the surface of the solution including the sample, and then the tip is mechanically contacted with the solid phase, thereby preparing a sample array. The capillary system using a capillary is such that the sample solution once sucked up to the capillary is mechanically contacted with the solid phase through the tip of the capillary as in the case of the pin system, thereby supplying the sample solution in an array form. For these

spotting operations, various commercially available apparatuses from various companies may be used. These methods are considered as most preferable in the sense that any sample DNA can be supplied. However, as for quantification, the problem may be in that viscosity varies depending on the length and concentration of DNA. For proteins, these methods are also preferred in the sense that they are deposited independently of the size and viscosity of molecules, but not suitable for quantitative analysis.

Outline of Sample Array Preparing Methods Through the Ink Jet Process

Samples capable of being discharged in an ink jet process include chemicals in addition to nucleic acids and proteins.

In the ink jet process, because a shearing force is exerted, the length of dischargeable nucleic acids and the size of dischargeable proteins are often limited. However, this process is superior in quantification to the pin system and capillary system, and is used more suitably than other systems with respect to the discharge of chemicals. Dischargeable nucleic acids are limited to those with a relative length to bases of 5 kb or smaller, and dischargeable proteins are limited to those of 1000 K daltons or less. As for chemicals, any chemicals can be discharged.

Any liquid can be used for discharging and supplying samples with ink jets, as long as this liquid is capable of being discharged from ink jets. The above-described liquid discharged from the head is shot to a predetermined position. When being mixed with nucleic acid probes and during discharge, the above-described nucleic acid probes are not damaged.

In terms of dischargeability from the ink jet, particularly from the bubble jet head, with respect to the

properties of the above-described liquid, it is preferable that its viscosity be in the range of 1 to 15 cps and its surface tension be 30 dyn/cm or larger. Also, if the viscosity is in the range of 1 to 5 cps and the surface tension is in the range of 30 to 50 dyn/cm, the positions in which the liquid is spotted on the solid phase are extremely accurate.

Therefore, if the stability of the nucleic acid during discharge or the like is taken into consideration, a nucleic acid probe of, for example, 2 to 5000 mer, particularly 2 to 1000 mer, is preferably contained in the solution at a concentration of 0.05 to 500 μM, particularly 2 to 50 μM.

FIG. 3 is a schematic explanatory view of a specimen solution discharging method through the bubble jet process, which is one embodiment of the present invention. In FIG. 3, reference numeral 101 denotes a liquid supplying system (nozzle) retaining a solution, including a specimen, as a discharge liquid in such a manner that the solution is capable of being discharged, reference numeral 103 denotes a solid phase having a nucleic probe bound thereto with which the above-described specimen is reacted, and reference numeral 105 denotes a bubble jet head that supplies heat energy to the above-described liquid to discharge it, and is a type of ink jet head. Reference numeral 104 denotes a liquid including the specimen discharged from the bubble jet head. FIG. 4 is a 4-4 line sectional view of the bubble jet head 105 in FIG. 3. In FIG. 4, reference numeral 105 denotes the bubble jet head. Reference numeral 107 denotes a liquid including a specimen solution to be discharged. Reference numeral 117 denotes a substrate portion having a heat generation portion to provide discharge energy to the above-described liquid. The substrate portion 117 includes a protective layer 109 formed from

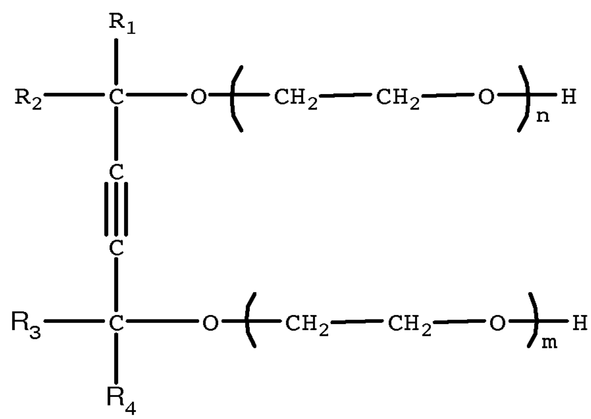
silicon oxide and the like, electrodes 111-1 and 111-2 formed from aluminum and the like, an exothermic resistor layer 113 formed from nichrome and the like, a heat storage layer 115, and a support 116 formed from aluminum having a good heat-release property.

The liquid 107, including the specimen, comes to a discharge orifice (discharge outlet) 119, and forms a meniscus 121 with a predetermined pressure. Here, when electric signals are applied to the electrodes 111-1 and 111-2, a region (foaming region) denoted by reference numeral 123 abruptly releases heat, and the liquid 117 contacted therewith is discharged and flies toward the solid surface 103. The amount of the liquid that can be discharged using a bubble jet head having such a structure varies depending on the size of its nozzle, but can be controlled approximately to 4 to 50 picoliters, which is extremely useful as a means for placing specimen probes at a high density.

In terms of dischargeability from the ink jet, particularly from the bubble jet head, with respect to the properties of the above-described liquid, it is preferable that its viscosity be in the range of 1 to 15 cps and its surface tension be 30 dyn/cm or larger. Also, if the viscosity is in the range of 1 to 5 cps and the surface tension is in the range of 30 to 50 dyn/cm, the positions in which the liquid is spotted on the solid phase are extremely accurate.

Therefore, if the stability of nucleic acid during discharge or the like is taken into consideration, a nucleic acid of, for example, 2 to 5000 mer, particularly 2 to 1000 mer, is preferably contained in the solution at a concentration of 0.05 to 500 μM, particularly 2 to 50 μM.

The composition of a discharged liquid is not particularly limited, as long as the liquid has no substantial influence on the nucleic acid probe when it is mixed with the nucleic acid probe and when it is discharged from the ink jet, and it can be normally discharged to the solid phase using the ink jet. Preferable liquids include glycerin, urea, thiodiglycol or ethylene glycol, isopropyl alcohol, and acetyl alcohol expressed by the following formula:



(I)

In the above formula (I), R_1, R_2, R_3 and R_4 represent alkyl groups, specifically linear or branched alkyl groups having 1 to 4 carbon atoms, m and n represent integer numbers, respectively, wherein m and n equal 0, or $1 \leq m + n \leq 30$, and if $m + n = 1$, m or n equals 0.

More specifically, a liquid containing 5 to 10% by weight (wt%) of urea, 5 to 10 wt% of glycerin, 5 to 10 wt% of thiodiglycol, and 0.02 to 5 wt%, more preferably 0.5 to 1 wt%, of acetylene alcohol presented by the above formula (I) may be suitably used.

The detecting method of the present invention is a method of detecting a complex formed between an oligonucleotide for detection probes and an object component, which is used for the purpose of making an evaluation/examination as to whether

or not a component capable of binding to the oligonucleotide and forming a complex in a liquid test sample for use as a detection probe whose base sequence is known, and as to the degree of binding capability thereof if such a component exists in the sample. For detecting this complex, the oligonucleotide for detection probes is fixed in advance on the solid surface substrate, whereby this fixed oligonucleotide is bound to the object component contained in the test sample, and the formed complex is separated while it is fixed on the solid substrate. On the basis of a methodology for detecting complexes using proper detecting means, the amount of test samples required at this time is reduced to a very low level. Also, the detection accuracy and sensitivity are kept at a sufficiently high level.

That is, in this methodology providing a base for the present invention, since surface density of the oligonucleotide for detection probes that is fixed on the solid surface substrate can be kept at a predetermined value, the amount of the formed complex is proportional to the binding capability of the object component, and is also proportional to the concentration of the object component contained in the test sample that is contacted with the solid surface substrate and is made to act on the oligonucleotide. Taking advantage of this characteristic, the test sample is contacted only with the surface, with the oligonucleotide for detection probes actually fixed thereon. The contact area is limited to a certain degree, whereby the amount of the used test sample is also limited to a certain degree. Specifically, by adopting a means for spotting a predetermined minimal amount of liquid in the form of droplets, the contact area and the amount of liquid put thereon are controlled with good reproducibility. The amount of the complex that would be

automatically fixed on the solid surface substrate with formation is detected for this limited contact area, thereby achieving detection accuracy and sensitivity that are essentially as high as those in the case of dipping the entire solid substrate with oligonucleotide for detection probes fixed thereon in the liquid test sample.

The complex is detected by the label bound on the surface of the substrate. When a complex of the oligonucleotide and the labeled test sample is formed and the individual spots are sufficiently spaced from each other, detection can be carried out independently for each spot. Therefore, if given or larger spaces are provided between adjacent spots, even though there are spots for different test samples nearby, only spots for desired test samples can be selected to continue detection work without being influenced by those spots. In the detection method of the present invention, in order to satisfy reliably, the requirement that given or larger spaces be provided between adjacent spots, a defined array is formed in the spot position as a result of providing predetermined spaces as spaces between spots, and a predetermined amount of sample solution is spotted for each spot to make the spot area (contact area) constant, or make the spot diameter constant to ensure reproducibility, because the shape of the spot (contact surface) is generally a circle. As a matter of course, for precluding the influence of adjacent spots, a space between spots is selected such that optical signals (fluorescent) and the like derived from the adjacent spots are not mixed in the detection system, in the light of the measured area (diameter in the measurement range) of the detection system selected as appropriate in accordance with the spot diameter. Also, as a matter of course, the detecting method of the present invention really shows its advantages in the case where there

exist two or more types of test samples, and they are detected simultaneously.

On the other hand, on the surface to which a plurality of spots of such array forms are provided, one type of oligonucleotide for detection probes should be fixed at a uniform surface density. Also, for the section in which the oligonucleotide for detection probes is fixed, its area and shape are selected as appropriate in accordance with the above-described array space and the total number of spots to be included in a series of arrays. It is also possible to provide sections having different oligonucleotides fixed therein in different regions on the detection substrate to be used, and to place a plurality of sections with two or more oligonucleotides fixed therein, respectively. That is, it can be said that the detecting method of the present invention becomes a more suitable method if used when two or more types of nucleotides are used as detection probes to carry out a series of evaluations simultaneously for a plurality of test samples, with respect to two or more types of object components corresponding to respective oligonucleotides.

Generally, in such an evaluation, it is often the case that the oligonucleotides for detection probes are predetermined while only an approximate number of test samples to be evaluated is determined. In such a case, it is preferable that as a detection substrate with oligonucleotides for detection probes fixed thereon in advance, a detection substrate with two or more types of detection probes put thereon systematically, having on the substrate surface in a matrix form sections in which respective oligonucleotides are fixed. In this detection substrate with fixed sections arranged thereon in a matrix form, the unit of a total number of spots that are made in an array form in each section is

fixed, but a plurality of these units can be used to carry out the evaluation depending on the number of test samples to be actually evaluated, thus enhancing convenience in practice. Furthermore, for each section arranged in a matrix form, a pattern formed by hydrophobic compounds is preferably provided in its substrate to provide a form in which mutual regions are separated from one another.

In the detecting method of the present invention, nucleic acid molecules may be selected as object components to apply the same when evaluating whether or not they are engaged in a double-strand formation in hybrid substances formed through a hybridization reaction with the oligonucleotide for detection probes. In this case, the method used is an effective method in which an evaluation is made at the same time even for multiple test samples as to whether or not nucleic acid molecules, including base sequences complementary to known base sequences of the oligonucleotide for detection probes, are contained in the test sample. Alternatively, if two or more types of nucleotides for detection probes are provided, and one type of nucleic acid molecules is contained in each test sample, an evaluation can be made for the nucleic acid molecule with the still unknown base sequences as to whether or not the nucleic acid molecule includes base sequences complementary to known base sequences of each oligonucleotide, which is effective, for example, as a means for searching for a gene group having a set of homologies.

The detection substrate of the present invention is a DNA probe substrate with oligonucleotides for use in probes respectively bound to sections arranged in a matrix form in advance. Particularly for the substrate, the bottoms of sections separated by wells (walls) of the frame structure matrix patterns formed in advance by hydrophobic compounds are

formed as hydrophilic surfaces, thereby making the binding of oligonucleotide easier. Also, by providing this hydrophobic wall, intermingling of DNA probes among adjacent sections can be curbed more reliably.

Also, using these DNA probe substrates, the test sample is spotted in an array form on the matrix of the oligonucleotide to carry out the hybridization reaction, thereby providing a means for checking quickly whether or not nucleic acid molecules with complementary sequences are included in each test sample for a certain oligonucleotide probe.

In this method, since the number of test samples that are used in the hybridization reaction is determined solely based on the number of spots, the size of the detection substrate is not limited. By using a substrate with a large area, the section in which each probe is fixed can be widened, and the necessity to increase density can be eliminated. Thus, since the section in which each probe is fixed can be increased, a wide range of methods can be used, such as methods in which a liquid containing probes is applied to, or printed as, a "solid printed pattern" through an ink jet process on defined regions on the substrate, or methods in which chemical synthesis is carried on the substrate, for means for binding of the probe/oligonucleotide on the substrate.

Also, considering that the probe/oligonucleotide is less expensive and easier to obtain than the test sample, no significant problem arises even if the area of the region to which oligonucleotide is bound is more or less increased. In this case, with respect to various kinds of test samples to be spotted, it is not necessary to always spot them at a high density. Furthermore, when the test sample is spotted in small amounts, the concentration of the object component that

is contained in the test sample is increased, whereby the hybridization reaction can be accelerated, making it possible to perform a highly sensitive detection for a short time. In addition, application of the detecting method of the present invention will open the door to fields that could not be considered previously, because a sufficient amount of samples could not be obtained, for example a new field in which mRNA obtained from tissues is directly examined.

Furthermore, information of reactivity in association with the obtained hybridization reaction is analyzed/evaluated in terms of existence/non-existence of complementarity to various kinds of oligonucleotides/probes, with respect to nucleic acid molecules contained in a specific test sample, thereby making it possible to carry out a detection that also functions similar to the conventional DNA arrays (hybridization reaction with multiple probes for one specimen).

Furthermore, the detecting method of the present invention provides a means for evaluating as object components the interaction between chemicals, in particular drugs and oligonucleotides, the bonding of proteins to oligonucleotides and the like. Therefore, it can also be used as a means for examining object components included in the test sample for multiple items, with respect to a large number of test samples. In addition, it provides a means for carrying out an examination on the same substrate at the same time and under the same conditions even for object components of different properties, such as chemicals, proteins and nucleic acids.

The detecting method of the present invention and the detection substrate for use exclusively therein will be described further in detail below.

FIG. 6 shows an example of applying the detecting method of the present invention to an embodiment in which cDNA is used as an object component. A hybrid substance is formed through a hybridization reaction with an oligonucleotide with known base sequence that is used for detection probes. In the detection substrate shown in FIG. 6, a plurality of rectangular sections separated systematically in a matrix form in advance are provided on the solid surface substrate in a rectangular form. The rectangular sections are each spatially isolated by matrix compartments that are surrounding walls. DNA probes that are used for hybridization/probes are each bound uniformly to the bottom surface of the rectangular sections.

Also, attached is an enlarged view showing schematically a situation in which a plurality of test samples including cDNA as an object component, for example two or more types of cDNA solutions prepared based on m-RNAs collected respectively are spotted in the form of the two-dimensional array like a square matrix, in a section with the DNA prove fixed therein. The detection substrate, the detection probe, components to be detected and the like that are used in the detecting method of the present invention will be described further in detail.

Oligonucleotides that are Used for Detection Probes

In the detecting method of the present invention, a deoxyribonucleic acid can be used for an oligonucleotide that is used for detection probes. In addition thereto, a ribonucleic acid, a peptide nucleic acid and the like can be used. Types thereof are not limited as long as they have desired base sequences, are capable of being bound to other molecules in those portions, and can be fixed on a solid substrate. Also, for portions excluding nucleic acid chains, those modified with non-nucleic acid atom groups and those

having additional structures and the like can also be used as long as the above-described requirements are satisfied.

Furthermore, for this oligonucleotide that is used for detection probes, a desired amount thereof should be artificially prepared or collected, and its base sequence itself should be known. However, its nucleic acid part should have at least two bases. Its base length is not limited in principle, but if the length exceeds that of 100 bases, it becomes significantly more difficult to use as its base length increases when fixing on the solid substrate is to be carried out. Therefore, the base length is preferably restricted to that of 100 bases or less.

For example, when this oligonucleotide is subjected to a hybridization reaction with, for example, nucleic acid molecules that are more than 100 bases long, the length of the oligonucleotide is preferably at least 10 mer for obtaining sufficient bonding. On the other hand, if the length exceeds 50 mer, it is difficult to set conditions for controlling the detection of mismatching, thus making it difficult to select and detect only those that are fully matched. Thus, in order to detect mutations, the length is preferably 60 mer or less.

Furthermore, the range of 10 mer to 60 mer is a preferable range even when the oligonucleotide having desired base sequences, for example DNA is prepared through chemical synthesis.

Shapes of Sections with Oligonucleotide Fixed Therein and Arranged in a Matrix Form

The shape of a section itself in which the oligonucleotide for detection probes is bound and fixed is not particularly limited. However, if a test sample is spotted in an array form on this section, generally a simpler shape rather than a complicated outside shape is preferably

selected. In addition, when the oligonucleotide is bound and fixed, generally, a simpler shape is preferably selected for providing a uniform surface density in such a section, in view of working efficiency and convenience. Specifically, rectangular forms, for example, line forms, squares and rectangles, are preferably adopted. Of course, in principle, forms whose perimeters are formed by curves, such as circles and ellipses, do not cause any problems.

On the other hand, in the detection substrate of the present invention, when two or more oligonucleotides that are used for detection probes are put on one substrate, sections in which they are fixed are preferably arranged in a matrix form, in view of working efficiency and convenience. Also, preferably, the form and area of each section is unified.

Density of Sections Arranged in a Matrix Form

The density of sections arranged in a matrix form is selected as appropriate depending on the number of oligonucleotides that are put on the detection substrate at a time. Moreover, the density of 400 per centimeter square or less is preferable. If the density is $400/cm^2$, and the form of each section is a square, the size of each section is a 500 μm square. If test samples are closely arranged in an array form as spots with diameters of 100 μm, 25 spots are arranged in total with 5 spots high by 5 spots wide. Also, if the diameter of the spot is 20 μm, the number of spots that can be arranged in a row is 25, leading to 625 spots in total. Since the detecting method of the present invention has more significant advantages when there is a large number of test samples and they are examined at the same time, the final object of the invention will be more satisfactorily achieved if the density of the section that is arranged is selected so that at least the upper limit of the number of test samples

that can be spotted approximately equals the above-described value.

For example, when the detecting method of the present invention is applied to test samples including cDNA, the number of test samples to be examined, specifically the total number of types of cDNA often is approximately as many as 3600. In this case, if the diameter of the spot is 100 μm, the size of one section approximately equals a 6 mm square when 60 spots are arranged in rows and columns, respectively. Also, even if the diameter of the spot is 20 μm, the size of one section should be a 1.2 mm square. In this way, in the detection substrate for use in the detecting method of the present invention, there are not a few cases of application objects where the density of sections that are arranged in a matrix form is preferably selected as 400 per centimeter square or less.

Furthermore, in the detecting method of the present invention, the test sample is spotted as droplets, and in the case where the diameter of the spot is 100 μm, for example, the amount of liquid required for the droplet of one spot is about 25 picoliters. Even if the number of probes for use in examination is selected as 400 (for example, the number of sections of the matrix to be provided on the substrate is 400) for this spot size, the total amount of liquid required for the whole spots may be no more than 10 nanoliters for each test sample, thus making it possible to carry out an objective examination with a minimal amount of liquid.

Also, in the conventional method, in which the detection substrate is dipped in the solution of the test samples, the amount of the required liquid depends on the size of the substrate. Thus, if the amount of the test sample is essentially very small, the size of the substrate should be

reduced in accordance with the amount of liquid, and it is essential to highly integrate probes that are fixed on the substrate. On the other hand, in the detecting method of the present invention, the size of the substrate itself can be freely selected without allowing for the liquid amount of the test sample. In addition, when the oligonucleotide that is used for detection probes is fixed, the surface density should be uniform as a matter of course. However, it is not necessary to highly integrate a plurality of probes to fix them, making the fixing operation easier.

Fixing of Oligonucleotide on the Substrate

As means for fixing the oligonucleotide that is used for detection probes on the surface of the substrate, a method in which the oligonucleotide separately prepared in advance is supplied in predetermined sections by coating or printing to bind the oligonucleotide, or a method in which each oligonucleotide, specifically, a DNA probe or the like is synthesized in a solid phase on the substrate to prepare originally bound DNA, can be used. Furthermore, even in the case where the oligonucleotide is not DNA, but, for example, is ribonucleic acid or peptide nucleic acid, synthesis on the substrate can be carried out to bind the oligonucleotide as described below.

On the other hand, when the oligonucleotide, specifically DNA or ribonucleic acid, peptide nucleic acid or the like, separately synthesized or collected in advance, is used for detection probes, a process of fixing the oligonucleotide by covalent bonding or by electrostatic coupling on the surface of the substrate can be used.

Synthesis of Oligonucleotide on the Substrate

Synthesis of DNA on the substrate includes synthesis on the silicon substrate using photolithography as a methodology

disclosed in U.S. Patent No. 5,445,934. This patent discloses a method in which high density DNA probe arrays are prepared by dividing the surface of the silicon substrate into very small areas and synthesizing DNA for probes. On the other hand, in the detection substrate for use in the detecting method of the present invention, for example, the size of the section in which each probe is fixed may be a 0.5 mm square or larger. Thus, it is not always necessary to enhance the density. However, also in the detection substrate of the present invention, photodecomposable protective groups, protective groups that are decomposed by chemicals and the like, are bound to the nucleic acid in advance, and processes of masking, light exposure and reaction are repeated, whereby DNA chains can be synthesized on each section using the methodology described in U.S. Patent No. 5,445,934 in which four types of nucleic acid bases are bound for each base to stretch the DNA chain having desired base sequences.

Fixing Oligonucleotide Synthesized or Collected in Advance

As means for carrying out the fixing using electrostatic coupling, a method in which polylysine, polyethyleneimine and polyalkylamineaone on the solid surface substrate are subjected to blocking using the negative charge of DNA is generally used.

However, in the case of the oligonucleotide with a base length of 60 or less, which is not sufficiently long, the electric charge of its phosphate groups is also weak. Thus, the binding with the substrate by the above-described method is not necessarily strong. For the oligonucleotide with a base length that is not sufficiently long, if a method in which an oligonucleotide with functional groups for a covalent bond introduced in the terminal of nucleic acid is synthesized

in advance, the substrate is subjected to surface processing suitable for functional groups, and the above-described functional groups are used to accomplish a covalent bond are used, stronger binding can be achieved, which is more preferable.

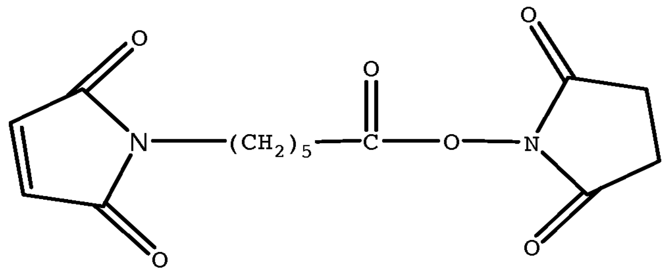
Also, in the case where the oligonucleotide is RNA, the above-described method, which is used for DNA, may be applied. Alternatively, in the case where the oligonucleotide is a peptide nucleic acid, its nucleic acid part may be used to apply the above-described method, which is used for DNA.

Types of Functional Groups for Use in Fixing by Covalent Bond Between the Solid Substrate and Oligonucleotide

When the oligonucleotide is fixed on the solid surface substrate though a covalent bond, functional groups are generally introduced in oligonucleotide and the solid surface substrate, respectively, in advance to carry out the reaction therebetween. For this combination of functions, a preferable example is a combination in which maleimide groups are introduced in the surface of the substrate and thiol groups (-SH) are introduced in the oligonucleotide. Specifically, thiol groups (-SH) are bound to the terminal of the oligonucleotide while the solid surface is subjected to processing of forming a coating having maleimide groups. When the oligonucleotide is supplied to the solid surface, the thiol groups (-SH) are made to act on and react with the maleimide groups to perform the fixing through the formation of a covalent bond.

For introducing maleimide groups in the solid surface, various kinds of methods may be used. For example, an aminosilane coupling agent is reacted with a glass substrate, and then a reagent (EMCS reagent: manufactured by Dojin Co., Ltd.), including N-(6- maleimidocaproyloxy) succinimide

expressed by the following formula, whereby a coating layer having maleimide groups can be formed.



As another example, a reagent containing succinimidyl 4-(maleimidophenyl)butyrate can be used to react with amino groups, preferably.

Also, for example, an oligonucleotide with thiol groups introduced therein can be synthesized by using 5' -Thiol-Modifier C6 (manufactured by Glen Research Co., Ltd.) as a five prime-end reagent when DNA is synthesized using a DNA automatic synthesizing apparatus. Furthermore, after synthesis, purification processing by high speed liquid chromatography is applied after a normal deprotection reaction.

Combinations of functional groups capable of being used for fixing by the covalent bond include, for example, a combination of epoxy groups (on the solid surface) and amino groups (the terminal of oligonucleotide) in addition to the above-described combination of thiol groups and maleimide groups. Methods for introducing epoxy groups in the solid surface include, for example, a method in which a coating is applied to the solid surface constituted by polyglycidyl methacrylate having epoxy groups and a method in which a silane coupling agent having epoxy groups is applied to the solid surface made of glass and is reacted with glass.

Supply of Oligonucleotide Solution by the Ink Jet Process

There is no particular limitation on the means for supplying predetermined sections on the surface of the solid substrate with a solution containing the oligonucleotide to be fixed thereon, as long as a uniform amount of liquid is supplied for each unit area. In the case where printing by the ink jet process and the like is used, a "solid print pattern" is prepared. Then, using an ink jet type printer head that is used for ink jet printers, the cartridge for the ink is filled with an oligonucleotide solution instead of the ink, and printing for a defined area is carried out. If the amount of the liquid to be supplied is small, items with a large volume, such as an ink cartridge, are not used. Instead, a structure in which a sample supplying portion, such as a tube, is connected to a head to supply the oligonucleotide solution to the head may be used.

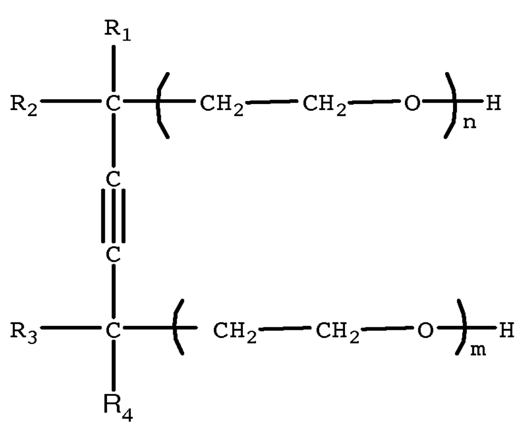
For the oligonucleotide solution for the discharge, which is used in this method, a solution that is capable of being discharged in the form of ink jets and has a viscosity suitable for a minimal amount of droplets discharged from the head to be shot onto a desired position is used. In addition, a solvent to be used is selected from solvents that satisfy the above-described requirements and do not damage the desired oligonucleotide in the state of being mixed with the desired oligonucleotide and during discharge.

Specifically, in terms of dischargeability from the ink jet head, particularly from the bubble jet head, it is preferable that, for example, the viscosity is in the range of 1 to 15 cps, and the surface tension is 30 dyn/cm or larger, as the properties of the solution. In particular, when the viscosity in the range of 1 to 5 cps and the surface tension in the range of 30 to 50 dyn/cm are selected, the position to which the solution is shot onto the substrate is extremely

accurate, and a supplying method using the bubble jet head is particularly suitably used.

In addition, in terms of the stability of the oligonucleotide during discharge and the like, the supplying means of the ink jet system is further preferred when, for example, a solution containing the oligonucleotide of 2 to 100 mer, particularly 2 to 60 mer, at concentrations ranging from 0.05 to 500 μM, preferably from 2 to 50 μM, is used.

In applying a discharging method of the ink jet system, the liquid composition of the oligonucleotide solution is not particularly limited, as long as the solution practically does not damage the desired oligonucleotide in the state of being mixed with the desired oligonucleotide and during discharge, as described above, and it can be discharged to the surface of the solid substrate using the ink jet. Furthermore, a preferable solution contains, for example, glycerin, urea, thiodiglycol or ethylene glycol, isopropyl alcohol, or acetylene alcohol expressed by the following formula in addition to desired oligonucleotide:



.

In the above formula, R^1, R^2, R^3 and R^4 represent alkyl groups, for example linear or branched alkyl groups having 1 to 4 carbon atoms, respectively, and m and n represent 0 or positive integer numbers, respectively, and satisfy $1 \leq m +$

n≤30. In addition, specifically, the liquid composition, including 5 to 10 wt% of urea, 5 to 10 wt% of glycerin, 5 to 10 wt% of thioglycol, and 0.02 to 5 wt%, more preferably 0.5 to 1 wt% of acetylene alcohol expressed by the formula (I), allows the discharging method of the ink jet system to be suitably used.

Structure of Matrices Composed of Hydrophobic Walls and Hydrophilic Wells

Also, for sections of the matrix that are provided on the solid surface, for example, sections of the matrix with hydrophobic walls (barriers) surrounding hydrophilic wells (recesses) may be formed to prevent coupling between adjacent sections. A structure may also be used in which the oligonucleotide solution is supplied to the hydrophilic wells (recesses) surrounded by the hydrophobic walls (barriers), and the oligonucleotide is fixed only in the bottom of the hydrophilic wells (recesses).

Materials of Walls/Wells

When the sections are arranged in a matrix form, the solution of oligonucleotide is supplied to the bottom of the wells (recesses) separated by wall (barrier) patterns to carry out the binding reaction. It is desirable that the bottom of the wells (recesses) is wetted densely with the solution, but the walls (barriers) have poor wettability with the solution. For example, it is preferable that the solid material constituting the surface of the bottom of the wells (recesses) is more hydrophilic, and the surface of the walls (barriers) and the portion corresponding to partitions with neighboring sections are less hydrophilic. The oligonucleotide solution supplied in the bottom of the well (recess) is spread across the bottom, but is prevented from finding its way over the wall (barrier) into adjacent sections. Also, even the droplet

erroneously supplied at the position related to the wall (barrier) quickly moves into a desired well (recess) having good wettability. As a result, a predetermined amount of the oligonucleotide solution can be supplied in the well (recess) more reliably.

An example of sections arranged in a matrix form that is provided on the detection substrate of the present invention is shown in FIG. 10. The sections in a square matrix form have a structure in which heights (walls) having frame structures are provided on the surface of the solid substrate, and arranged rectangular recesses (wells) are separated. Specifically, the recesses (wells) separated from one another by the heights (walls) having frame structures are formed by coating the entire surface of the solid substrate with a material forming heights (walls), and thereafter providing rectangular through-holes (cut-off portions) to open recesses (wells). Thus, the bottom of the recess (well) has an exposed surface of the solid substrate. The exposed portion of the surface of the solid substrate is subjected to processing for providing a surface to which the oligonucleotide can be bound. As a result, the oligonucleotide is fixed only in the bottom of this recess (well).

Materials forming heights (walls) having frame structures include, for example, metals (chrome, aluminum, gold, etc.) and resins. Resins include, for example, acryl, polycarbonate, polystyrene, polyimide, acrylate monomers and urethane acrylate, and photosensitive resins, such as photoresists, having black dies and black pigments contained therein. Furthermore, for specific examples of photosensitive resins, UV resists, DEEP-UV resists, ultraviolet cured resins and the like can be used. UV resists may include negative resists, such as cyclized polyisoprene-aromatic bisazide

resists, phenol resin-aromatic azide compound resists, and positive resists, such as novolac resin-diazonaphthoquinone resists.

DEEP-UV resists may include, for example, radiation dispersion type polymer resists, such as polymethyl methacrylate, polymethylene sulfone, polyhexafluorobutyl methacrylate, polymethyl isoprobenil ketone and bromo poly 1-trimethylcylilpropine, and dissolution inhibiting resists, such as cholate o-nitrobenzyl ester as positive type resists, and may include borovinylphenol-3-3'- diazidediphenylsulfone, and polymethacrylate glycidyl as negative type resists.

Ultraviolet cured resins may include polyester acrylate, epoxy acrylate and urethane diacrylate containing approximately 2 to 10% by weight of one or more types of photopolymerization initiators, which are selected from benzophenone and substituted derivatives thereof, oxime compounds, such as benzyl, and so on.

When detection is carried out using a fluorescent mark, a light-blocking material can be used effectively for curbing a light reflex by the material forming this height (wall) having a frame structure. For providing a light-blocking property, it is effective to add black pigments in the above-described resins. In this case, black pigments that can be used may include carbon black and black organic pigments.

Furthermore, if the height (wall) having a frame structure is formed by the above-described hydrophobic resin, the surface of the height (wall) is hydrophobic. The configuration in which heights (walls) having frame structures that are formed by hydrophobic materials are provided is more preferable when an aqueous solution is used as a solution containing the oligonucleotide to be supplied to the surface of the substrate of recesses (wells). Even if the aqueous

solution is supplied in a position related to the surface of the height (wall), it is not persistently attached to the surface of the wall, but gradually moves to the bottom of the recess (well) located in a lower position. Also, solutions of different oligonucleotides are supplied to adjacent recesses (wells), but they are separated from each other by the hydrophobic height (wall). Therefore, intermingling (cross-contamination) between solutions due to the penetration of the liquid is prevented.

Furthermore, for the thickness (height from the solid surface) of the height (wall) having a frame structure, the volume of the recess (well) is selected in view of the amount of the oligonucleotide solution that is supplied to the recess (well), and the thickness is determined as appropriate so that the volume is filled with the solution. Also, depending on methods of forming the height (wall), the thickness is preferably selected such that it is in the range of 1 to 20 μm and satisfies the above-described requirement. The thickness of the height (wall) selected in this way is in the range of the thickness allowing to effectively prevent cross-contamination between adjacent wells when the oligonucleotide solution is supplied to each well by the ink jet process.

Types of Specimens

Object components contained in the test sample to which the detecting method of the invention can be applied include mRNA, cDNA, proteins, cell extracts and chemicals, such as drugs.

Furthermore, when cDNA is used as an object component, it is possible to use double-strand cDNA directly. However the single-strand cDNA marked in advance is preferable in forming hybrid substances efficiently and performing detection thereof conveniently.

On the other hand, mRNA is essentially single stranded, and it is marked in some way to form marked mRNA, thereby making it possible to form hybrid substances efficiently and to perform detection thereof. Furthermore, the amount of mRNA in the test sample is generally small, and it is an object component more remarkably reflecting the advantage that the amount of sample solution required for detection can be reduced to a low level, which is characteristic of the detecting method of the present invention. However, since RAN decomposition enzymes tend to admix during handling, a predetermined amount of a substance to prevent decomposition of mRNA, such as RNA decomposition enzyme inhibitors, such as diethyl pyrocarbonate, is desirable added in the test sample solution. In addition to mRNA, similarly, the genome of RNA viruses can be an object component. In addition, tRNA, ribosomal RNA and the like can be object components.

On the other hand, when the protein is used as an object component, formed complexes can be detected using the fluorescence emitted by the protein itself.

Also, some chemicals emit their own fluorescence, enabling formed complexes to be detected using the fluorescence. Chemicals that do not emit fluorescence may be marked by methods using functional groups of compounds. Those to which the detecting method of the invention can be applied may include, for example, chemicals that can be bound to single-stranded DNA. In addition, they may include, for example, chemicals that can be bound to single-stranded RNA.

Means for Spotting Test Samples in an Array Form

In the detecting method of the present invention, the test sample is spotted in an array form in a defined position on the detection substrate. For the purpose of reducing the amount of the required liquid to a minimal level, the spot

diameter is selected so that it is in the range of several tens to 100 μm. However, with such a spot diameter, the liquid should be spotted in high uniformity of spotted amounts and high positional accuracy. As a means for satisfying this requirement, spotting apparatuses of pin systems, ink jet systems and capillary systems may be used.

The pin system refers to a method in which the test sample is attached to the pin tip, and the end point thereof is mechanically contacted with the solid surface, thereby taking out a fixed amount of the test sample. The capillary system using capillaries refers to a method in which the test sample solution is sucked up to the capillary on a temporary basis, and the tip of the capillary is mechanically contacted with the solid surface as in the case of the pin system, thereby taking out a fixed amount of the test sample. Various kinds of spotting apparatuses adopting these two systems are commercially available. Thus, commercially available apparatuses may be used.

The spotting apparatuses of the pin system and capillary system enable any types of test samples to be spotted, and are considered as the most preferable methods for unknown test samples. For example, however, the viscosity of the test sample solution is varied depending on the length and the concentration of DNA contained in the test sample. Therefore, the amount of the spotted liquid varies. Thus, a problem arises in terms of quantification. Also, with respect to proteins, the viscosity of the test sample solution varies depending on the size of the molecules and the concentration, thus raising a problem in terms of quantification.

Spots in an Array Form of Test Samples by the Ink Jet Process

Specimens that can be discharged by the ink jet process include chemicals in addition to nucleic acids and proteins.

In the ink jet process, because a shearing force is exerted, the length of nucleic acids and the size of proteins that can be discharged are limited. However, it is superior in quantification to the pin system and capillary system, and is used more suitably than other systems, particularly with respect to the discharge of chemicals. Preferably, dischargeable nucleic acids are those with a relative length to bases of 5 kb or smaller, and dischargeable proteins are those of 1000 K daltons or less. As for chemicals, their molecular weights are generally small enough compared to nucleic acids and proteins. Therefore, any chemical can be discharged, except for polymers having extremely large molecular weights.

FIG. 3 illustrates schematically a method of discharging specimen solution by the ink jet process, particularly the bubble jet process, which is one means that is used for spotting test sample solution in the present invention. In FIG. 3, reference numeral 101 denotes a liquid supply system (nozzle) retaining a solution including a specimen as a discharge liquid in such a manner that the solution is capable of being discharged, reference numeral 103 denotes a solid phase having a nucleic probe bound thereto with which the specimen is reacted, and reference numeral 105 denotes a bubble jet head for providing heat energy to the liquid to discharge it, which is a type of ink jet head. Reference numeral 104 denotes a liquid (droplet) including the specimen discharged from the bubble jet head. FIG. 4 is a sectional view of the bubble jet head 105 described in FIG. 3. In FIG. 4, reference numeral 107 denotes a liquid including a specimen solution to be discharged from the bubble jet head 105, and

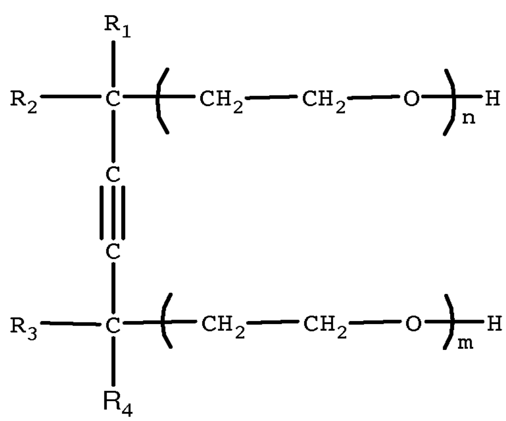
reference numeral 118 denotes a substrate portion having a heat generation portion to provide discharge energy to the above-described liquid. The substrate portion 118 includes a protective layer 109 formed by silicon oxide and the like, electrodes 111-1 and 111-2 formed by aluminum and the like, an exothermic resistor layer 113 formed by nichrome and the like, a heat storage layer 115, and a support 116 formed by aluminum having good heat-release properties. The liquid 107 including the specimen comes to a discharge orifice (discharge outlet) 119 and forms a meniscus 121 with a predetermined pressure. In this situation, when electrical signals are applied to the electrodes 111-1 and 111-2, a region (foaming region) denoted by reference numeral 123 abruptly releases heat, and the liquid 117 contacted therewith is discharged and flies toward the solid surface 103. The amount of liquid that can be discharged using a bubble jet head having such a structure varies depending on the size of its nozzle, but can be controlled to approximately 4 to 50 picoliters, which is extremely useful as a means for arranging probes at a high density in a matrix form on the surface of the substrate.

In terms of dischargeability from the ink jet, particularly from the bubble jet head, for the properties of the above-described liquid, it is preferable that its viscosity be in the range of 1 to 15 cps and its surface tension be 30 dyn/cm or larger. Also, if the viscosity is in the range of 1 to 5 cps and the surface tension is in the range of 30 to 50 dyn/cm, the position in which the droplet is spotted (spot position) on the solid phase is extremely accurate.

In addition, if the stability of nucleic acid during discharge or the like is taken into consideration, a single-stranded nucleic acid or double-stranded nucleic acid of, for

example, 2 to 5000 mer, particularly 2 to 10000 mer, is preferably contained in the solution. For example, c-DNA chips are preferably contained at the concentration of 0.05 to 500 μM, particularly 2 to 50 μM.

The composition of liquid is not particularly limited, as long as the liquid has no substantial influence on the nucleic acid probe when it is mixed with the nucleic acid probe and when it is discharged from the ink jet. The liquid can be normally discharged to the solid phase using the ink jet. Preferable are liquids including glycerin, urea, thiodiglycol or ethylene glycol, isopropyl alcohol, and acetyl alcohol expressed by the following formula:



.

In the above formula, R^1, R^2, R^3 and R^4 represent alkyl groups, specifically linear or branched alkyl groups having 1 to 4 carbon atoms, m and n represent 0 or positive integer numbers, respectively, and satisfy $1 \leq m + n \leq 30$.

Further, specifically, a liquid containing 5 to 10% by weight (wt%) of urea, 5 to 10 wt% of glycerin, 5 to 10 wt% of thiodiglycol, and 0.02 to 5 wt%, more preferably 0.5 to 1 wt% of acetylene alcohol, is suitably used.

Examples

The present invention will be described in detail below using Examples. Furthermore, the Examples shown herein

represent most suitable embodiments of the present invention, and the invention should not be limited by these Examples.

Example 1

A glass substrate with black matrices for specimen matrices for analyzing sequences of p 53 genes on a specimen matrix substrate partitioned by patterns is prepared.

1. Preparation of a black matrix introduction substrate coated with polylysine.

A glass substrate (60 mm x 50 mm) made of synthetic quartz is subjected to supersonic cleaning using 2% sodium hydrate solution, and is then subjected to UV ozone processing to clean the surface. Then, a polylysine solution (manufactured by Sigma Co., Ltd.) is applied to the entire surface with a spin coater. In addition, a DEEP-UV resist (negative type resist for black matrices) (BK-739P manufactured by Nippon Steel Chemical Co., Ltd.) is applied thereto with the spin coater so that the thickness after curing is 5 μm, and this substrate is heated for curing at 80°C for 5 minutes with a hotplate. Using a DEEP-UV aligner, a region of 1 cm x 1 cm is proximately exposed to light using a patterned mask, so that the distance (X) between adjacent wells in FIG. 1 is 100 μm and the form of the well is a 1 mm x 1 mm square, and then development is carried out with a developing inorganic alkaline solution using a spin drier, and the developing solution is washed out completely with purified water.

Then, the substrate is briefly dried using the spin drier, and is thereafter heated at 180°C for 30 minutes in a clean oven to have the resist fully cured to obtain a substrate in which 400 wells are at a predetermined arrangement and adjacent wells are separated from each other

by the black matrix. Furthermore, the volume of each well is calculated as 5 µl if the thickness of the liquid is 5 µm.

2. Fixing Specimen DNA

(1) Preparation of cDNA libraries

The p 53 gene is obtained by a PCR reaction from 64 types of cDNA libraries obtained form tumor tissues.

That is, RNA samples were obtained from each tissue collected with biopsies using Catrimox-14 (Biotechnology Co., Ltd.). Based on this sample solution, First-Strand cDNA Synthesis Kit (manufactured by Life Sciences Co., Ltd) is used to obtain cDNA libraries.

(2) Amplification of p53 genes having T3 binding sites by a PCR method

Based on the cDNA library, "Human p53 Amplimer set" manufactured by CLONTECH Co., Ltd. is used to carry out the PCR reaction.

As a PCR reaction solution, "one shot LA PCR Mix" (Takara Shuzo Co., Ltd.) was used. The composition of the PCR reaction solution is as follows:

One shot LA PCR Mix	25 µl
5' primer (20 µM)	1
3' primer (20 µM)	1
cDNA library solution	1
DW	22/50 µl.

The PCR cycle is such that after thermal denaturation at 95°C for 5 minutes, cycles at 95°C for 30 seconds, at 55°C for 30 seconds and at 72°C for 60 seconds are conducted 29 times. Finally, the solution is left for a reaction at 72°C for 5 minutes and is then stored at 4°C.

After the reaction, gel electrophoresis is performed to confirm a product existing in the region of molecular weight

of about 300 mer, and purification is carried out with MicroSpin Column S200 (Pharmacia) to obtain p 53 genes (p 53 DNA).

(3) Synthesis of single-stranded p 53 DNA

Using as a matrix the DNA obtained in the above (2), a single-stranded marked DNA is obtained by the PCR reaction using 5' primer (Takara Shuzo Co., Ltd.). The composition of the reaction solution comprises

One shot LA PCR Mix	25 μl
5' primer (20 μM)	1
P 53 DNA	1
DW	22/50 μl, and

the reaction cycle is such that cycles at 96°C for 30 seconds, at 60°C for 15 seconds and at 60°C for 4 minutes are repeated 24 times, and finally, the solution is stored at 4°C. Thereafter, it is purified with MicroSpin Column S200.

(4) Fixing p 53 cDNA

5 μl of the single-stranded DNA obtained in the above (3) is injected under a microscope into each well of the polylysine-coated substrate with black matrices prepared in the above (1), and is fixed through electrostatic coupling.

3. Analysis of variation of p 53 genes with oligonucleotide probes

The 64 DNAs were selected, focusing the attention on the 248th and 249th amino acid sequences of the p 53 gene being a tumor inhibitor gene. That is, it is known that a case of frequent variation in the base sequence of CGGAGG is the case where the first C is changed to T, the second A is changed to G, and the third G of the sequence corresponding to the 249th amino acid is changed to T. Thus, the 64 probes are designed,

focusing the attention on the base sequence at these three points.

That is, it is a structure in which the total length of the probe is 18 mer, and six bases including this variation are located at the center thereof, and the bases are sandwiched between common sequences. A common sequence corresponds to the range from the five prime-end to the ATGAAC, and the subsequent portion including variation corresponds to the NNGAGN and a further subsequent common portion corresponds to the CCCATC, resulting in a final sequence of 5'ATGAACNNGAGNCCCATC3' (SEQ ID NO:65). Here, the portion expressed by N corresponds to the A, G, C, and T that are four types of nucleic acid bases. The probe DNA has a sequence complementary to the sequence to be detected (the above-described sequence), and thus the sequence thereof is 5'GATGGGNCTCNNGTTCAT3' (SEQ ID NO:66). Rhodamine is coupled to the five prime-end of each probe sequence to mark the prove. Specific base sequences of these 64 types of marked DNA probes are shown in the following Table 1.

Table 1

SEQ ID NO.	Sequence	SEQ ID NO.	Sequence
1	GATGGGACTCAAGTTCAT	33	GATGGGCCTCAAGTTCAT
2	GATGGGACTCAGGTTCAT	34	GATGGGGCTCAGGTTCAT
3	GATGGGACTCACGTTCAT	35	GATGGGCCTCACGTTCAT
4	GATGGGACTCATGTTCAT	36	GATGGGCCTCATGTTCAT
5	GATGGGACTCGAGTTCAT	37	GATGGGCCTCGAGTTCAT
6	GATGGGACTCGGGTTCAT	38	GATGGGCCTCGGGTTCAT
7	GATGGGACTCGCGTTCAT	39	GATGGGCCTCGCGTTCAT
8	GATGGGACTCGTGTTCAT	40	GATGGGCCTCGTGTTCAT
9	GATGGGACTCCAGTTCAT	41	GATGGGCCTCCAGTTCAT
10	GATGGGACTCCGGTTCAT	42	GATGGGCCTCCGGTTCAT
11	GATGGGACTCCCGTTCAT	43	GATGGGCCTCCCGTTCAT
12	GATGGGACTCCTGTTCAT	44	GATGGGCCTCCTGTTCAT
13	GATGGGACTCTAGTTCAT	45	GATGGGCCTCTAGTTCAT
14	GATGGGACTCTGGTTCAT	46	GATGGGCCTCTGGTTCAT
15	GATGGGACTCTCGTTCAT	47	GATGGGCCTCTCGTTCAT
16	GATGGGACTCTTGTTCAT	48	GATGGGCCTCTTGTTCAT
17	GATGGGGCTCAAGTTCAT	49	GATGGGTCTCAAGTTCAT
18	GATGGGGCTCAGGTTCAT	50	GATGGGTTCTAGGTTCAT
19	GATGGGGCTCACGTTCAT	51	GATGGGTCTCACGTTCAT
20	GATGGGGCTCATGTTCAT	52	GATGGGTCTCATGTTCAT
21	GATGGGGCTCGAGTTCAT	53	GATGGGTCTCGAGTTCAT
22	GATGGGGCTCGGGTTCAT	54	GATGGGTCTCGGGTTCAT
23	GATGGGGCTCGCGTTCAT	55	GATGGGTCTCGCGTTCAT
24	GATGGGGCTCGTGTTCAT	56	GATGGGTCTCGTGTTCAT
25	GATGGGGCTCCAGTTCAT	57	GATGGGTCTCCAGTTCAT
26	GATGGGGCTCCGGTTCAT	58	GATGGGTCTCCGGTTCAT
27	GATGGGGCTCCCGTTCAT	59	GATGGGTCTCCCGTTCAT
28	GATGGGGCTCCTGTTCAT	60	GATGGGTCTCCTGTTCAT
29	GATGGGGCTCTAGTTCAT	61	GATGGGTCTCTAGTTCAT
30	GATGGGGCTCTGGTTCAT	62	GATGGGTCTCTGGTTCAT
31	GATGGGGCTCTCGTTCAT	63	GATGGGTCTCTCGTTCAT
32	GATGGGGCTCTTGTTCAT	64	GATGGGTCTCTTGTTCAT

Then, for each of the 64 types of marked prove DNAs, a 8 μM solution containing glycerin, urea and thiodiglycol at the final concentration of 7.5%, and acetylenol EH at the final concentration of 1% is prepared. A different probe solution is charged by 100 μl in each of the six nozzles of BJ Printer Head BC 62 (manufactured by Canon Inc.). An arrangement is made so that six DNAs can be discharged for each head, and two heads are used to discharge 12 DNAs at a time, and the heads are exchanged 6 times to discharge DNAs so that each spot of 64 DNA is formed independently. In this manner, a total of 64 probes are discharged in the form of the 8 x 8 array in each well of a black matrix coated with polylysine.

FIG. 5 shows an arrangement on each black matrix of 64 DNA probes that are discharged. In this case, 64 DNA probes are spotted in one matrix.

Thereafter, this substrate in which each probe is spotted is left in a humidifier chamber set at 40°C to carry out a hybridization reaction.

Thereafter, the substrate is cleaned with a 10 mM phosphate buffer containing 100 mM NaC1 to remove DNA probes that have not been engaged in the formation of the hybrid substance.

DNA arrays after the hybridization reaction are observed using an inverted fluorescence microscope equipped with a filter set suitably for rhodamine.

If the gene as a specimen has normal base sequences, spots of highest fluorescence intensity should be observed in the gene at the location of the relative 42nd DNA prove. It can be considered that those are derived from the hybrid of the p 53 gene having normal sequences exposed to the probe DNA and PCR. In a varied gene, detectable spots are observed at

the location other than the 42nd location, and a varied sequence can be identified from the DNA probe supplied to the location.

Example 2

Evaluation of Existence/Non-existence of Carcinogenic Genes Using mRNA

1. Extraction of mRNA

"QuickPrep Micro mRNA Purification Kit” (manufactured by Amersham Pharmacia Biotech co., Ltd.) is used to extract mRNA from tumor tissues collected with the biopsy. This mRNA is bound to a polylysine substrate with black matrices, as in the case of Example 1.

2. Examination of existence/non-existence of carcinogenic genes and the type thereof with various kinds of carcinogenic gene probe arrays

Sets of cloned oncogenes (18 types, manufactured by Takara Shuzo Co., Ltd.) are purchased. Then, "LabelITnon-RI Labeling Kits" are used to perform rhodamine marking.

18 types of marked oncogene probes are spotted as an arrangement of 4 x 5 on the above-described substrate with mRNA bound thereto, using a microarray preparing apparatus (pin system) manufactured by Cartesian Technologies Co., Ltd.

Further, a hybridization reaction is carried out as in Example 1.

The type of oncogenes existing in the mRNA section extracted from each tissue can be known.

At this time, sufficient detection can be performed with one type of marks irrespective of the types of oncogenes present.

The second aspect of the invention is described more specifically below with reference to the Examples.

Example 3

An example of procedures for preparing a substrate with oligonucleotide bound thereto will be described below. In this embodiment, a detection substrate with the oligonucleotide bound to a region of 2 mm square on a glass substrate was prepared in accordance with the procedure described below.

1. Cleaning of the substrate

A glass substrate of 1-inch square was placed on a rack and soaked in a detergent for ultrasonic cleaning. Thereafter, it was subjected to ultrasonic cleaning in the above-described detergent for 20 minutes, followed by removing the detergent by rinsing. Also, it was rinsed with distilled water, followed by further performing ultrasonication for 20 minutes in a container containing distilled water.

Then, this glass substrate was soaked for 10 minutes in 1N sodium hydrate solution heated in advance. After it was taken out from the solution, the 1N sodium hydrate solution adhered to the surface was washed out with water. Thereafter, cleaning with distilled water was continued.

2. Surface treatment

The above-described cleaned glass substrate was soaked in an aqueous solution of 1% silane coupling agent (manufactured by Shin-Etsu Chemical Co., Ltd., Trade name: KBM 603) at room temperature for 20 minutes, followed by spraying nitrogen gas on both sides of the substrate to drive off water for drying. The substrate was baked for one hour by using an oven heated to 120°C to complete the treatment of the surface of the glass substrate with a silane coupling agent.

On the other hand, 2.7 mg of EMCS (N-(6-Maleimidocaproyloxy) succinimide: manufactured by Dojin Co., Ltd.) was weighed, and was dissolved in a solution of

DMSO/ethanol (1:1) (final concentration of 0.3 mg/ml). The glass substrate subjected to the treatment with a silane coupling agent was soaked in this EMCS solution for two hours to carry out the reaction between the amino group of the silane coupling agent covering the surface of the substrate and the succinimide group in the EMCS solution. In association with this reaction, the substrate is covered with EMCS through the silane coupling agent. In the obtained glass surface, a maleimide group derived from the EMCS is present on the surface. The glass substrate taken out after the reaction with the EMCS solution is cleaned with distilled water, and is thereafter dried with nitrogen gas. This glass substrate subjected to the surface treatment for introducing a maleimide group will be used for a binding reaction with DNA described below.

3. Synthesis of DNA for fixing glass substrates

An oligonucleotide having a base sequence of the following Sequence 1 (SEQ ID NO:42) is chemically synthesized for fixing on the glass substrate. This sequence 1 is a 18 mer sequence including in its central part a base sequence with a base length of 6 to code 248th and 249th amino acids in an amino acid sequence of a gene product (peptide chain) that is coded by the p 53 gene known as a tumor suppressor gene. Also, A SH group is introduced in its 5' end for fixation on the glass substrate.

Sequence 1 5' HS-GATGGGCCTCCGGTTCAT3' (SEQ ID NO:42)

The SH group is introduced by using a commercially available reagent Thiol-Modifier (manufactured by GlenResearch Co., Ltd.) on a DNA automatic synthesizing apparatus. Subsequently, normal deprotection was carried out to recover DNA. The DNA was purified by high-speed liquid chromatography, and was then used in the following processes.

4. Discharging of DNA using a BJ printer head and binding thereof to a substrate

The above-described synthetic oligonucleotide (DNA) was dissolved in water, and the solution was diluted to a concentration of 8 μM using SG Clear (a solution containing 7.5% of glycerin, 7.5% of urea, 7.5% of thiodiglycol and 1% of acetylenol EH).

100 μl of this oligonucleotide solution was charged into the nozzle of BJ printer head BC 62 (manufactured by Canon Inc.) with the nozzle modified so that it is suitable for a small amount of samples (discharged amount). This modified printer head was set in a plotting apparatus to perform printing over the surface of the glass substrate as an area of "solid print" of 2 mm square with the oligonucleotide solution. Furthermore, the modified printer head that was used is used for bubble jet type ink jet printing and enables printing to be performed at a resolution of 360 x 720 dpi.

Thereafter, the glass substrate coated with the oligonucleotide solution was left in a humidifier chamber for 30 minutes to carry out a reaction between the maleimide group on the surface of the substrate and the thiol group (HS-) of the oligonucleotide. Thereafter, the unreacted oligonucleotide was removed. The prepared substrate for detection has the synthetic DNA (oligonucleotide) of the above-described Sequence 1 bound to a predetermined 2 mm square section on the glass substrate via a covalent bond.

Example 4

Supply of cDNA solution to the Surface of the Substrate with Oligonucleotide Bound Thereto and Hybridization Reaction

From various kinds of cDNA libraries obtained from tumor tissues, p 53 gene fragments were PCR-amplified, and then only

one type of side chains was reamplified using primers marked in advance to prepare marked single-stranded cDNA for use as test samples. The hybridization reaction was carried out between this marked single stranded DNA derived from the p 53 gene and the DNA probe bound on the detection substrate prepared in Example 3.

1. Preparation of test samples

From 64 types of cDNA libraries obtained from tumor tissues, p 53 gene fragments were obtained by the PCR reaction.

Specifically, first, all RNA samples were separated/collected from respective tissues collected via a biopsy, using Catrimox-14 (Biotechnology Co., Ltd.). On the basis of all the RNA sample solutions, a c-DNA library was prepared using First-Strand cDNA Synthesis Kit (manufactured by Life Science Co., Ltd.). A primer for amplifying p 53 genes was added to this cDNA library to amplify P 53 gene fragments. With this PCR amplification product as a template, the marked five-side primer was used to carry out the PCR reaction (DNA synthetic reaction) to amplify only one type of side chains. By this amplification, marked single stranded DNA derived from the p 53 gene can be prepared.

(1) Amplification of p 53 gene fragments having a T3 binding site in the terminal by the PCR method

For using a primer for auto sequencers (Takara Shuzo Co., Ltd) using T3 promoters as the above-described marked primer, a primer having a T3 site in the terminal and having coupled to its downstream a base sequence allowing the p 53 gene part to be amplified was first synthesized. The PCR reaction was carried out using this primer to obtain a PCR amplification product having a T3 promoter site coupled to the p 53 gene part.

In this example, for the five prime-end primer for amplifying p 53 genes, the primer with a base sequence having a T3 promoter site coupled to its five side (T3-P53), was prepared. The base sequence is shown below.

5'
AATTAACCCTCACTAAAGGGAACCTGAGGTTGGCTCTGACTGTACCACCATCC3' (SEQ ID NO:67)

In the sequence, the underlined part on the side of five prime-end represents a T3 polymerase binding site. On the other hand, for a three prime-end primer for amplification, a three prime-end primer attached in a commercially available amplification kit, "Human p 53 Amplimer Set" of CLONTECH Co., Ltd., was used. For a PCR reactive solution, "one shot LA PCR Mix" (Takara Shuzo Co., Ltd.) was used.

The solution composition in the PCR reaction has:

one shot LA PCR Mix	25 μl
T3-P53 primer (20 μM)	1 μl
3' primer (20 μM)	1 μl
cDNA library solution	1 μl
DW	22 μl/50 μl.

For the PCR cycle, the cycles were conducted at 95°C for 30 seconds, at 55°C for 30 seconds and at 72°C for 60 seconds at 29 times after thermal denaturation at 95°C for 5 minutes, and finally keeping the solution at 72°C for five minutes. The reactant was stored at 4°C on a temporary basis after it was cooled.

After the reaction, gel electrophoresis was carried out to confirm a PCR product present in the region of molecular weight of about 300 mer. This PCR product was purified with Micro Spin Column 5200 (Pharmacia) to obtain p 53 gene fragments to which the T3 primer can be coupled (T3-linked p 53 DNA).

(2) Synthesis of marked single-stranded DNA using labeled T3 primers (Rho-T3)

With the p 53 gene fragment obtained in (1) as a matrix, single stranded marked DNA was obtained with the PCR reaction, using a Rho-T3 primer (Takara Shuzo Co., Ltd.). The composition of the reactive solution had:

one shot LA PCR Mix	25 μl
Rho-T3primer (10 μM)	1 μl
T3-linked p 53 DNA	1 μl
DW	23 μl/50 μl.

For the reaction cycle, the cycles were conducted at 96°C for 30 seconds, at 50°C for 15 seconds and at 60°C for 4 minutes 24 times. The reactant was stored at 4°C on a temporary basis after it was cooled. It was purified with Micro Spin Column S200. Thereafter, gel electrophoresis was carried out to confirm desired rhodamine labeled single-stranded DNA synthesized through the PCR reaction.

2. Supply of test sample solution

Sodium chloride was added in the test sample obtained in the above-described process, namely the solution of rhodamine-marked single-stranded DNA derived from the p 53 gene, so that the final concentration of the solution was 1M. The solution of rhodamine-marked single-stranded DNA derived from the p 53 gene, which had been prepared from 64 types of c DNA libraries, was injected into each well of a 96-hole microtiter plate. These solutions of rhodamine-marked single-stranded DNA were spotted in an 8 x 8 arrangement onto the detection glass substrate with the DNA probe of Sequence 1 obtained in Example 3 in the form of 2 mm square, using a microarray preparing apparatus (pin system) manufactured by Cartesian Technologies. The diameter of each spot was 100 µm.

3. Hybridization reaction

This detection substrate with a total of 64 types of rhodamine-marked single-stranded DNA solutions being sample specimens spotted thereon was left in a humidifier chamber set at 40°C to carry out a hybridization reaction for 3 hours. Thereafter, the detection substrate was washed with a 10 mM phosphate buffer containing 100 mM NaCl to remove test samples that had not been engaged in the formation of hybrid substances.

After the hybridization reaction, the test sample spotted in the form of a two-dimensional 8 x 8 array was observed using an inverted fluorescence microscope equipped with a filter set for excitation light and fluorescence suitable for fluorescence marked rhodamine. For most of the spots, red fluorescence derived from fluorescence marked rhodamine in association with the formation of hybrid substances was observed. However, fluorescence intensity was weak for six spots and no fluorescence was observed for one spot.

Due to this, it can be considered that since in the p 53 gene derived from corresponding six types of tumor cells variation occurs somewhere in the base sequence corresponding to the 248th and 249th of the amino acid sequence of the p 53 gene product (p 53 protein), the amount of formed hybrid substances is small due to its mismatch. In that connection, the fluorescence intensity from the fluorescence mark is weak. For the test sample in which fluorescence was not observed, it can be considered that because hybrid substances in p 53 cDNA fragments did not form, a deficiency occurs in the base sequence to code the above-described 248th and 249th amino acid sequence. Consequently, hybrid substances could not be formed.

Example 5

Preparation of Array form Spots of Test Samples on the Probe Matrix Detection Substrate with Multiple Oligonucleotides Fixed Thereon

1. Preparation of 64 probe matrices

Processing was performed as in Example 3 to prepare a glass substrate having a maleimide group. 64 DNAs with the base sequences as shown in Table 2 were printed (applied) thereon in the area of 2 mm square, respectively, using a bubble jet printer head similar to that in Example 3 to prepare a detection substrate on which sections with 64 types of prove DNAs fixed therein were arranged in a matrix form.

Focusing the attention on the 248th and 249th amino acids of the amino acid sequence of the gene product (p 53 protein) of the p 53 gene being a tumor suppressor gene, 64 DNAs with base sequences as shown in Table 1 were selected on the basis of the base sequence to code these two amino acids so that a sequence with various kinds of base variations added thereto was obtained. Specifically, it is known that a case of frequent variation in the base sequence CGGAGG providing a base is the case where the first C of the CGG to code the 248th amino acid is changed to T, the second A is changed to G, and the third G of the AGG to code the 248th amino acid is changed to T. Thus, 64 probes were designed to provide sequences capable of being bound to base sequences with these bases at three positions varied in various ways.

Specifically, it was a structure in which the total length of the probe was 18 mer, six bases including this variation were located in the center thereof, and common base sequences with base lengths of 6 were placed before and after the six bases. More specifically, the structure has a common sequence of ATGAAC from the side of the five prime-end, the

base sequence of NNGAGN as a portion including the variation, and a common sequence of CCCATC on the side of three prime-end.

It was a base sequence complimentary to the sequence of 5'ATGAACNNGAGNCCCATC3'(SEQ ID NO:65). That is, it was a probe expressed by 5'GATGGGNCTCNNGTTCAT3' (SEQ ID NO:66). Furthermore, since it is a DNA probe, the portion denoted by N in the above-described base sequence refers to any one of A, G, C and T that are four DNA nucleic acid bases.

Table 2

1	5'-GATGGGACTCAAGTTCAT-3'	33	5'-GATGGGCCTCAAGTTCAT-3'
2	5'-GATGGGACTCAGGTTCAT-3'	34	5'-GATGGGCCTCAGGTTCAT-3'
3	5'-GATGGGACTCACGTTCAT-3'	35	5'-GATGGGCCTCACGTTCAT-3'
4	5'-GATGGGACTCATGTTCAT-3'	36	5'-GATGGGCCTCATGTTCAT-3'
5	5'-GATGGGACTCGAGTTCAT-3'	37	5'-GATGGGCCTCGAGTTCAT-3'
6	5'-GATGGGACTCGGGTTCAT-3'	38	5'-GATGGGCCTCGGGTTCAT-3'
7	5'-GATGGGACTCGCGTTCAT-3'	39	5'-GATGGGCCTCGCGTTCAT-3'
8	5'-GATGGGACTCGTGTTCAT-3'	40	5'-GATGGGCCTCGTGTTCAT-3'
9	5'-GATGGGACTCCAGTTCAT-3'	41	5'-GATGGGCCTCCAGTTCAT-3'
10	5'-GATGGGACTCCGGTTCAT-3'	42	5'-GATGGGCCTCCGGTTCAT-3'
11	5'-GATGGGACTCCCGTTCAT-3'	43	5'-GATGGGCCTCCCGTTCAT-3'
12	5'-GATGGGACTCCTGTTCAT-3'	44	5'-GATGGGCCTCCTGTTCAT-3'
13	5'-GATGGGACTCTAGTTCAT-3'	45	5'-GATGGGCCTCTAGTTCAT-3'
14	5'-GATGGGACTCTGGTTCAT-3'	46	5'-GATGGGCCTCTGGTTCAT-3'
15	5'-GATGGGACTCTCGTTCAT-3'	47	5'-GATGGGCCTCTCGTTCAT-3'
16	5'-GATGGGACTCTTGTTCAT-3'	48	5'-GATGGGCCTCTTGTTCAT-3'
17	5'-GATGGGGCTCAAGTTCAT-3'	49	5'-GATGGGTCTCAAGTTCAT-3'
18	5'-GATGGGGCTCAGGTTCAT-3'	50	5'-GATGGGTTCTAGGTTCAT-3'
19	5'-GATGGGGCTCACGTTCAT-3'	51	5'-GATGGGTCTCACGTTCAT-3'
20	5'-GATGGGGCTCATGTTCAT-3'	52	5'-GATGGGTCTCATGTTCAT-3'
21	5'-GATGGGGCTCGAGTTCAT-3'	53	5'-GATGGGTCTCGAGTTCAT-3'
22	5'-GATGGGGCTCGGGTTCAT-3'	54	5'-GATGGGTCTCGGGTTCAT-3'
23	5'-GATGGGGCTCGCGTTCAT-3'	55	5'-GATGGGTCTCGCGTTCAT-3'
24	5'-GATGGGGCTCGTGTTCAT-3'	56	5'-GATGGGTCTCGTGTTCAT-3'
25	5'-GATGGGGCTCCAGTTCAT-3'	57	5'-GATGGGTCTCCAGTTCAT-3'
26	5'-GATGGGGCTCCGGTTCAT-3'	58	5'-GATGGGTCTCCGGTTCAT-3'
27	5'-GATGGGGCTCCCGTTCAT-3'	59	5'-GATGGGTCTCCCGTTCAT-3'
28	5'-GATGGGGCTCCTGTTCAT-3'	60	5'-GATGGGTCTCCTGTTCAT-3'
29	5'-GATGGGGCTCTAGTTCAT-3'	61	5'-GATGGGTCTCTAGTTCAT-3'
30	5'-GATGGGGCTCTGGTTCAT-3'	62	5'-GATGGGTCTCTGGTTCAT-3'
31	5'-GATGGGGCTCTCGTTCAT-3'	63	5'-GATGGGTCTCTCGTTCAT-3'
32	5'-GATGGGGCTCTTGTTCAT-3'	64	5'-GATGGGTCTCTTGTTCAT-3'

Then, for each of the 64 types of labeled prove DNAs, an 8 μM solution containing glycerin, urea and thiodiglycol at the final concentration of 7.5%, respectively, and acetylenol EH at the final concentration of 1% was prepared. As in

Example 4, using BJ Printer Head BC 62 (manufactured by Canon Inc), a different DNA probe solution was charged by 100 μl in each of the six nozzles of the printer head. Using a plurality of such printer heads, a detection substrate with total 64 DNA probes applied to and fixed in each section of 2 mm square in the form of a "solid print" and arranged in a matrix form (8 x 8) was prepared. A schematic layout of the 64 DNA probes arranged in a matrix form (8 x 8) on the detection substrate is shown in FIG. 7.

2. Preparation of array spots of test samples.

As in the case of Example 4, 64 types of labeled cDNAs were spotted in the form of the two-dimensional 8 x 8 array on each region of 2 mm square for fixing probes. Specifically, as schematically shown in FIG. 8, a pin system array preparing apparatus was used to form spots in the form of the two-dimensional 8 x 8 array on the sections arranged in a matrix form (8 x 8) in which each DNA probe was fixed.

3. Hybridization reaction

A hybridization reaction was carried out using conditions and procedures similar to those in Example 4. The result thereof is shown in FIG. 9. In the arrangement shown in FIG. 7, with respect to spots on probes corresponding to the base sequence of the 42nd normal gene, fluorescence intensity was weak for six spots as in Example 4. Also, no fluorescence was observed for one spot. In addition thereto, it was observed that fluorescence was emitted from the spot at three points in the tenth probe region, at two points in the 41st probe region, and at one point in the 46th probe region, respectively.

Spot positions in which fluorescence in association with the formation of hybrid substances was observed in the prove

region having these base sequences including variations corresponded to spot positions of weak fluorescence intensity in the probe region having the above-described 42nd original base sequence. Thus, if the base sequences of the probes are compared between both regions, the base sequence of the tenth probe is ACTCCG, the base sequence of the 41st probe is the CCTCCA, and the base sequence of the 46th probe is CCTCTG with respect to the original base sequence of CCTCCG of the 42nd probe. For their complementary sequences, it can be understood that with respect to CGGAGG in the 42nd probe, the CGGAGT and G were changed to T in the tenth probe, the TGGAGG and C were changed to T in the 41st probe, and the CAGAGG and G were changed to A in the 46th probe. That is, it was confirmed that in test samples, forming hybrid substances with these tenth, 41st and 46th probes, cDNA fragments contained therein derived from the p53 gene caused one base mismatch with respect to the 42nd probe due to the above-described variations.

By this method, existence/non-existence of variations and types thereof could be detected at the same time for all the 64 types of test samples.

Example 6

Preparation of a Substrate for Probe Matrices Partitioned by Patterns

A glass substrate with an epoxy group introduced to the surface and with black matrices for probe matrices was prepared in accordance with the following procedure.

1. Introduction of an epoxy group to the surface of the substrate

A glass substrate made of synthetic quartz (50 mm x 50 mm) was first subjected to ultrasonic cleaning using a 2% sodium hydrate solution, and was then subjected to UV ozone

processing to clean the surface. A 50% methanol solution containing 1% of a silane coupling agent (trade name: KBM 403; manufactured by The Shin-Etsu Chemical Co., Ltd.) containing a silane compound having an epoxy group bonded thereto (γ-glycidoxypropyltrimethoxysilane) was stirred at room temperature for three hours to perform a preliminary treatment for hydrolyzing the methoxy group in the silane compound. This solution already subjected to the hydrolysis treatment was applied to the surface of the above-described clean substrate with a spin coater, and was heated and dried at 100°C for 5 minutes to form a binding coating of the silane coupling agent on the surface of the substrate. Through the formation of this coating, the epoxy group contained in the silane compound was introduced to the surface of the substrate.

2. Formation of black matrices

Then, A DEEP-UV resist containing carbon black (negative type resist for black matrices) (trade name: BK-739P; manufactured by Nippon Steel Chemical Co., Ltd.) was applied on the surface of the substrate with a spin coater, so that the film thickness after curing was 5 μm, and it was heated for curing on a hotplate at 80°C for 5 minutes. By the proximity exposure using a DEEP-UV aligner, a pattern was exposed to light using as an exposure mask a mask for negatives with patterning applied to a region of 10 mm x 10 mm, so that the distance X between adjacent wells was 100 µm and the outer shape of the well was a 1 mm x 1 mm square. Then, the development was carried out with an inorganic aqueous alkaline developer solution using a spin drier, and the substrate was washed with pure water to completely remove the developer. Then, it was briefly dried using the spin

drier, and was thereafter heated in a clean oven at 180°C for 30 minutes to fully cure the resist. As a whole, a substrate with 400 wells in a predetermined arrangement and black matrices (resist walls) partitioning adjacent wells was obtained. Furthermore, the internal volume of each well is calculated as 5 μl if the thickness of the solution is 5 μm. Also, in the surface of the prepared black matrix, the angle of contact to water was 93 degrees and wettability with water was significantly low, while in the bottom of the well, the angle of contact to water was 35 degrees and the wettability with water was high.

3. Fixing probe DNA

64 oligonucleotides of 18 mer with an amino group bound to the hydroxyl group of the five prime-end through a phosphate group and hexamethylene were prepared as DNA probes. The 64 probes are same as those prepared in Example 5 with respect to base sequences, but are different in the sense that an amino group is introduced in their five prime-end instead of a thiol group.

5 μl of solution of these DNA probes was injected into each well under a microscope, and was left in a humidified chamber to allow the probe to bind to the substrate through the reaction between the amino group of the five primer-end and the epoxy group on the substrate.

Example 7

Analysis of cDNA derived from the p 53 gene that has been prepared from mRNA, using the probe matrix substrate partitioned by the pattern that has been prepared in Example 6 is as follows.

As in the case of Example 4, 64 types of labeled cDNAs were spotted in each probe region of 2 mm square as an

arrangement of 8 x 8 spots, as shown in FIG. 8, using a pin system array preparing apparatus.

A hybridization reaction was carried out by a method similar to that in Example 4.

The obtained result was similar to that in Example 5.

SEQUENCE LISTING

<110>Canon INC.
<120>An assay of many samples for multiple items at the same time
<130>3912041
<160>64
<210>1
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>1
gatgggactc aagtt cat
<210>2
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>2
gatgggactc aggtt cat
<210>3
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>3
gatgggactc acgtt cat
<210>4
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>4
gatgggactc atgtt cat
<210>5
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>5
gatgggactc gagtt cat

<210>6
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>6
gatgg gactc gggtt cat
<210>7
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>7
gatgggactc gcgttcat
<210>8
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>8
gatgggactc gtgttcat
<210>9
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>9
gatgggactc cagttcat
<210>10
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>10
gatgggactc cggttcat
<210>11
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>11

gatgggactc ccgttcat
<210>12
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>12
gatgggactc ctgttcat
<210>13
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>13
gatgggactc tagttcat
<210>14
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>14
gatgggactc tggttcat
<210>15
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>15
gatgggactc tcgttcat
<210>16
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>16
gatgggactct tgttcat
<210>17
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide

<400>17
gatggggctc aagttcat
<210>18
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>18
gatggggctc aggttcat
<210>19
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>19
gatggggctca cgttcat
<210>20
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>20
gatggggctc atgttcat
<210>21
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>21
gatggggctcg agttcat
<210>22
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>22
gatggggctc gggttcat
<210>23
<211>18
<212>DNA
<213>Artificial sequence
<220>

<223>Sample oligonucleotide
<400>23
gatggggctc gcgttcat
<210>24
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>24
gatggggctc gtgttcat
<210>25
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>25
gatggggctc cagttcat
<210>26
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>26
gatggggctc cggttcat
<210>27
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>27
gatggggctc ccgttcat
<210>28
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>28
gatggggctc ctgttcat
<210>29
<211>18
<212>DNA
<213>Artificial sequence

<220>
<223>Sample oligonucleotide
<400>29
gatggggctct agttcat
<210>30
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>30
gatggggctc tggttcat
<210>31
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>31
gatggggctc tcgttcat
<210>32
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>32
gatggggctc ttgttcat
<210>33
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>33
gatgggcctc aagttcat
<210>34
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>34
gatgggcctc aggttcat
<210>35
<211>18
<212>DNA

<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>35
gatgggcctc acgttcat
<210>36
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>36
gatgggcctc atgttcat
<210>37
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>37
gatgggcctc gagttcat
<210>38
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>38
gatgggcctc gggttcat
<210>39
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>39
gatgggcctc gcgttcat
<210>40
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>40
gatgggcctc gtgttcat
<210>41
<211>18

<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>41
gatgggcctc cagttcat
<210>42
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>42
gatgggcctc cggttcat
<210>43
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>43
gatgggcctc ccgttcat
<210>44
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>44
gatgggcctc ctgttcat
<210>45
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>45
gatgggcctc tagttcat
<210>46
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>46
gatgggcctc tggttcat
<210>47

<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>47
gatgggcctc tcgttcat
<210>48
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>48
gatgggcctc ttgttcat
<210>49
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>49
gatgggtctc aagttcat
<210>50
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>50
gatgggtctc aggttcat
<210>51
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>51
gatgggtctc acgttcat
<210>52
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>52
gatgggtctc atgttcat

<210>53
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>53
gatgggtctc gagttcat
<210>54
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>54
gatgggtctc gggttcat
<210>55
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>55
gatgggtctc gcgttcat
<210>56
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>56
gatgggtctc gtgttcat
<210>57
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>57
gatgggtctc cagttcat
<210>58
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>58

gatgggtctc cggttcat
<210>59
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>59
gatgggtctc ccgttcat
<210>60
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>60
gatgggtctc ctgttcat
<210>61
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>61
gatgggtctc tagttcat
<210>62
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>62
gatgggtctc tggttcat
<210>63
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>63
gatgggtctc tcgttcat
<210>64
<211>18
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide

<400>64
gatgggtctc ttgttcat
<210>65
<211>18
<212>DNA
<213>Artificial sequence
<220>
<221> misc_feature
<222> (7)..(8)
<223> n is A, G, C or T
<220>
<221> misc_feature
<222> (10)..(10)
<223> n is A, G, C or T
<400>65
atgaacnnga gncccatc 18
<210>66
<211>18
<212>DNA
<213>Artificial sequence
<220>
<221> misc_feature
<222> (7)..(7)
<223> n is A, G, C or T
<220>
<221> misc_feature
<222> (11)..(12)
<223> n is A, G, C or T
<400>66
gatgggnctc nngttcat 18
<210>67
<211>53
<212>DNA
<213>Artificial sequence
<220>
<223>Sample oligonucleotide
<400>67
aattaaccct cactaaaggg aacctgaggt tggctctgac tgtaccacca tcc 53